The natural transformation in Staphylococcus aureus

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Abstract

Staphylococcus aureus (*S. aureus*) is a commensal bacterium which behaves as an opportunistic pathogen. Its genome possesses various kinds of mobile genetic elements (MGE) carrying pathogenic factors and antibiotic resistance genes. This thesis focuses on one of the horizontal gene transfer (HGT) systems, the natural transformation, which had not been demonstrated for a long time in *S. aureus*.

This thesis consists of four chapters. Chapter 1 is the general introduction, especially focusing on the *S. aureus* natural transformation that is triggered by the key regulator, sigma factor H (SigH). In Chapter 2, I demonstrate the natural transformation does not require any phage components, excluding the argument that natural transformation might be due to the phage-dependent "pseudo-competence". In Chapter 3, the environmental factors affecting the natural transformation efficiency are described, especially about the effect of an autolysis inhibitor, Sodium Polyanethol Sulfonate (SPS). In Chapter 4, I discuss the significance of the natural transformation in the evolution of *S. aureus* including the acquisition of drug-resistant genes.

Index

List of Abbreviation

AMR: antimicrobial resistance
B. subtilis: *Bacillus subtilis*
BHI: brain heart infusion
CRP: cyclic AMP receptor protein
CFU: colony forming unit
Cm: chloramphenicol
CS: complete synthetic media
DNA: deoxyribonucleic acids
dsDNA: double-strand DNA
E. coli: *Escherichia coli*
ECF: extra-cytoplasmic function
EDTA: ethylenediaminetetraacetic acid
Erm: erythromycin
GFP: green fluorescent protein
HGT: horizontal gene transfer
H. influenzae: *Haemophilus influenzae*
H. pylori: *Helicobacter pylori*
IR: inverted repeat
kbp: kilo base pair
Km: kanamycin
L. lactis: *Lactococcus lactis*
LB: lysogeny broth
NB: nutrient broth
N. gonorrhoeae: *Neisseria gonorrhoeae*
MGE: mobile genetic elements
MLST: multilocus sequencing typing
MRSA: methicillin-resistant *S. aureus*
MSSA: methicillin-sensitive *S. aureus*
OD: optical density
PBS: phosphate buffered saline

PCR: polymerase chain reaction

RNA: ribonucleic acid

S. aureus: *Staphylococcus aureus*

S. pneumoniae: *Streptococcus pneumoniae*

SCC: staphylococcal cassette chromosome

SCC*mec*: staphylococcal cassette chromosome *mec*

SDS: sodium dodecyl sulfate

SigA: Sigma factor A

SigB: Sigma factor B

SigH: Sigma factor H

SigS: Sigma factor S

SPS: sodium polyanethol sulfonate

ssDNA: single-strand DNA

ST: sequence type

S. pyogenes: *Streptococcus pyogenes*

Tet: tetracycline

TSA: TSB-agar

TSB: trypticase soy broth

UV: ultraviolet

WHO: World Health Organization

V. cholera: *Vibrio cholerae*

Chapter 1

Introduction

1-1. *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*), first discovered and described over 130 years ago (Ogston. 1882), belongs to the low G+C % Gram-positive Bacilli class of *Firmicutes* that also includes *Bacillus subtilis* (*B. subtilis*) and *Streptococcus pneumonia* (*S. pneumonia*). Known as a commensal bacterium, often colonizing mammalian nasal cavities (Wertheim et al. 2005), *S. aureus* is also a major human pathogen causing a broad spectrum of infections ranging from food poisoning and superficial skin abscesses to more serious diseases such as pneumonia, meningitis, osteomyelitis, septicemia, toxic shock syndrome and sepsis (Lowy. 1998). It has acquired resistance to a wide variety of antibiotics (Lowy. 2003; Ito et al. 2003), and methicillin-resistant strains (MRSA), the most common cause of nosocomial infections, are now spreading into the community (Chambers and Deleo. 2009).

The *S. aureus* genome contains several mobile genetic elements (MGE) such as transposons, bacteriophages, insertion sequences, pathogenicity islands and a staphylococcal cassette chromosome (SCC) (Lindsay. 2010; Malachowa and DeLeo. 2010). SCC carries many of the toxin and antibiotic resistance genes such as enterotoxin, and *mec* encoding resistance to any types of β-lactam antibiotics including methicillin. Whole genome sequencing analysis of *S. aureus* genomes revealed that closely clustered organisms have remarkably different MGE profiles, indicating frequent transfer and loss of MGE (Lindsay. 2014; Lindsay. 2010). Thus, horizontal gene transfer (HGT) plays a critical role in the evolution of this human pathogen.

1-2. The horizontal gene transfer in *S. aureus*

In bacteria, HGT is known to be mediated by three major mechanisms; conjugation, transduction, and natural transformation (Figure 1). Conjugation is cell-to-cell DNA transfer via the bridge encoded by conjugative plasmids. Conjugation

requires a series of *tra* genes, which are only found in limited parts of isolates in *S. aureus* (Novick. 1991). Transduction is mediated by phages. Most *S. aureus* strains are lysogenized with temperate phages, which can enter a lytic cycle that leads to generalized transduction, i.e., any part of the host DNA mispackaged into the phage particle is transferred to a recipient cell upon the following infection. In addition, unusual phage-like infectious particles are involved in the efficient transfer of staphylococcal pathogenicity islands (Novick et al. 2010).

Natural genetic competence for transformation involves the binding and uptake of extracellular DNA by the DNA incorporation machinery (also known as competence machinery) expressed at the cell surface (Claverys et al. 2009). Following a publication in 1972 reporting the existence of a transformation-like phenomenon in *S. aureus* (Lindberg et al. 1972), numerous reports investigating this process appeared over the ensuing decade. It was finally shown that this was in fact not natural genetic competence, but a type of HGT that requires contaminating phage tail fragments in the DNA preparation, which bind to the host cell and allow entry of DNA (Birmingham and Pattee. 1981). This unique DNA transfer in staphylococci is now termed "pseudo-transformation" or "pseudo-competence, which will be demonstrated to be distinct from the genuine natural competence in this thesis.

1-3. Competence genes for natural transformation

Orthologues of competence genes encoding the DNA uptake machinery are conserved in staphylococcal genomes (Kuroda et al. 2001; Morikawa et al. 2003) (Figure 2). Competence machinery consists of proteins encoded by *comG*, *comE* and *comF* operons in *Firmicutes* bacteria (Chen and Dubnau. 2004; Claverys et al. 2009; Fontaine et al. 2015).

The *comG* operon, first discovered in *S. pneumoniae*, encodes three genes, *comGA*, *comGB* and *comGC* (Albano et al. 1989; Blokesch. 2016). These three genes are required for the transport of transforming DNA in *S. pneumoniae* and *B. subtilis* (Peterson et al. 2004; Chen et al. 2006). In these bacteria, ComGC (*comGC*) compose the essential part of transformation pilus, which allows the exogenous double-strand DNA (dsDNA) to access to the dsDNA receptor (ComEA) (Provvedi and Dubnau. 1999; Chen and Dubnau. 2004). ComGA (*comGA*) is known as an ATPase localized

on the cytoplasmic side of the membrane, while ComGB (*comGB*) is a polytopic membrane protein (Chen and Dubnau. 2004; Chung et al. 1998). *S. aureus* possesses homologous genes to c*omGA*, *comGB* and *comGC* (Laurenceau et al. 2013; Cehovin et al. 2013). In addition to these three genes, the staphylococcal *comG* operon contains three additional open reading frames (*comGD*, *comGE* and *comGF*) encoding minor pilin subunits (Mann et al. 2013). Processing of the pre-pilins to mature pili requires the ComC signal peptidase (Chen and Dubnau. 2004; Chen et al. 2006; Fagerlund et al. 2014).

The *comE* operon consists of three genes, *comEA*, *comEB*, and *comEC*. ComEA (*comEA*) is the dsDNA receptor, which presumably delivers dsDNA to a protein that generates single-strand DNA (ssDNA) for internalization (Provvedi and Dubnau. 1999; Chen and Dubnau. 2004). ComEC (*comEC*) forms a channel for the internalized DNA (Burton and Dubnau. 2010). The role of ComEB (*comEB*) is still unclear; the deficient mutants in *B. subtilis* show a normal transformation rate. *S. aureus* and *B. subtilis* possess those there genes, while *S. pneumoniae* has only the *comEA* and *comEC* genes (Provvedi and Dubnau. 1999; Peterson et al. 2004).

The *comF* operon consists of the *comFA*, *comFB*, and *comFC* genes in *B. subtilis*. *S. aureus* and *S. pneumoniae* possess only *comFA*, and *comFC* genes. ComFA (*comFA*) and ComFC (*comFC*) are thought to form the machinery for the transfer of ssDNA into the cytoplasmic membrane. ComFA is located on the inside of the cytoplasmic membrane and controls the rate of DNA uptake. (Takeno et al. 2011; Fagerlund et al. 2014). ComFB (*comFB*) assembles with the DNA uptake machinery at the cell poles in *B. subtilis* (Kaufenstein et al. 2011; Fagerlund et al. 2014), but its function is still unclear.

In addition, other competence related factors are also involved in natural transformation. ssDNA binding proteins (Ssb, DprA, RecA, and CoiA) are responsible for the protection of DNA from intracellular degradation in *S. pneumoniae* (Fontaine et al. 2015). *S. aureus* possesses homologous genes encoding Ssb, DrpA, RecA, CoiA.

1-4. RNA polymerase Sigma factor H (SigH) to transcribe the genes for competence machineries in *S. aureus*

RNA polymerase consists of core enzyme subunits ($\alpha_2\beta\beta'$) that engages in all transcription, and one of sigma factors that recognize the specific promoter sequences (Wösten. 1998). Most bacteria possess multiple kinds of sigma factors. Each sigma factor recognizes distinct consensus promoter sequences, allowing bacteria to express different sets of genes. The sigma factors are grouped into two families, the σ^{70} and the σ^{54} families, that share little sequence similarity. The majority of the sigma factors belong to σ^{70} family that is further divided into subfamilies.

In *S. aureus*, four σ^{70}-family sigma factors have been identified. SigA, σ^{A}, is conserved as the primary sigma factor in all bacteria (Deora et al. 1997). SigB, σ^{B}, is a homologue of SigB of *B. subtilis* (Wu et al. 1996; Kullik and Giachino. 1997). SigH, the focus of this thesis, was found in 2003 (Morikawa et al. 2003). SigS, σ^{S}, is similar to the extra-cytoplasmic function (ECF) sigma factors (Shaw et al. 2008; Burda et al. 2014).

Staphylococcal SigH has a unique evolutionary characteristic in that it shares exceptionally low sequence similarity between different bacterial species (Morikawa et al. 2003; Morikawa et al. 2008). It belongs to a large group also including SigH of *B. subtilis*, and ComX of *S. pneumoniae*. These related sigma factors are widely distributed among *Firmicutes*, with diverse physiological roles: in *B. subtilis*, SigH (Spo0H) is required for transcription of early sporulation genes (Jaacks et al. 1989; Predich et al. 1992) whereas in *S. pneumoniae*, ComX (SigX) directs the expression of late genetic competence genes in response to a peptide quorum-sensing regulatory pathway (Lee and Morrison. 1999; Luo et al. 2003; Luo and Morrison. 2003). Staphylococcal SigH is responsible for transcription of the *comG* and *comE* operons (Morikawa et al. 2003).

1-5. SigH expression mechanisms

Although its artificial overexpression induces expression of the *comE* and *comG* operons, the *sigH* gene appears to be cryptic since its expression could not be detected under standard laboratory culture conditions (Morikawa et al. 2003). Because true bacterial cryptic genes are more akin to pseudogenes, likely to be lost through “use it or lose it” evolutionary constraints (Hall et al. 1983; Tamburini and Mastromei. 2000),

the conservation within *S. aureus* strains of SigH and the competence gene orthologues it controls suggested that they must be expressed and play a role under certain specific growth conditions.

We had reported two distinct SigH expression mechanisms (Morikawa and Takemura et al. 2012) (Figure 3). SigH expresses via the transient duplication of *sigH*, generating a chimeric gene with the downstream gene (Kugelberg et al. 2006). The duplication unit as well as the chimeric gene partner can vary among SigH active clones. The emergence frequency of SigH expressing cells by this mechanism is less than 10^{-5}. Another SigH expression mechanism involves post-transcriptional regulation, and independent of the duplication mechanism described above. The post-transcriptional regulation involves the 13-base inverted-repeat (IR) sequence lying just upstream of the start codon of *sigH*. This second mechanism requires specific growth conditions. SigH-dependent production of GFP reporter is induced under aerobic growth in a complete synthetic medium (CS2 medium; Table 1), while anaerobic conditions are essential in other synthetic media (CS1 medium, -GS medium). Thus, environmental factors seem to be important for the SigH expression.

1-6. DNA transfer into SigH expressing cells

Using CS2 medium and the strain over-expressing SigH, our lab developed a protocol through which reproducible DNA transfer can be achieved (Morikawa and Takemura et al. 2012). The plasmid pT181 was transferred at the frequency of 10^{-9}. We also demonstrated the transfer of chromosomal 52 kilo base pair (kbp) SCC*mec*II element. Since the *comE* and *comG* operon were essential for this transfer (Less than 10^{-10}), it was considered to be the natural transformation. Nevertheless, considering the long historical arguments in terms of “pseudo-transformation”, and the impact of the finding of staphylococcal transformation, there remained a challenge to demonstrate that SigH-dependent DNA transfer does not require any phage components.

1-7. This thesis

This thesis focuses on the SigH-dependent DNA transfer, and the environmental factors modulating it.

Chapter 2 focuses on the transformability of SigH active cells without phage factors. Plasmid DNA transformation was possible without any trace of the bacterial phage, demonstrating the genuine transformation. Genomic DNA transformation was also confirmed without phage factors.

Chapter 3 focuses on environmental conditions affecting the SigH-dependent natural transformation. The importance of cell wall metabolism was implicated by a series of experiments using the SigH-expressing cells.

In Chapter 4, I discuss the significance of natural transformation in *S. aureus* evolution, especially focusing on the transformation of large size DNA, and its impact on the emergence of multi-drug resistant bacteria.

Chapter 2

Sigma Factor H Dependent Natural Competence for DNA Transformation in *Staphylococcus aureus*

2-1. Abstract

In this chapter, the requirement of phage factors for sigma factor H (SigH) mediated transformation was investigated. As the result, SigH mediated transformation of plasmid DNA did not require the phage factors, indicating that *Staphylococcus aureus* (*S. aureus*) cells expressing SigH develop the genuine natural transformation. Genomic DNA was also transformable without phage factors. Taken together with the previous findings, a unique model for staphylococcal competence regulation by SigH was proposed, which could help explain the acquisition of antibiotic resistance genes through horizontal gene transfer (HGT) in this important pathogen.

2-2. Introduction

An alternative sigma factor SigH is responsible for transcription of the *comG* and *comE* competence operon orthologues in *S. aureus* (Morikawa et al. 2003) (Chapter 1). Transformation protocol was developed using SigH expressing cells and complete synthetic medium, CS2, and both plasmid DNA and genome DNA were successfully transferred (Morikawa and Takemura et al. 2012). This transfer required *comE* operon and *comG* operon genes, suggesting that it is through the natural genetic competence. However, the strain initially used for the transformation assay carried a lysogenic phage. Considering the long arguments in 1970's regarding the phage dependent pseudo-transformation, and the impact to propose the natural transformation in *S. aureus* for the first time, it was necessary to demonstrate that the SigH dependent DNA transfer is distinct from the phage dependent transduction or pseudo-transformation. To achieve this, this chapter aimed to establish a DNA transfer system that does not contain any phage component.

2-3. Material and Methods

2-3-1. Bacterial strains and culture conditions

S. aureus strains and oligonucleotides used in this study are listed in Table 2 and 3. *S. aureus* was grown in Brain Heart Infusion (BHI) medium, Trypticase Soy Broth (TSB), Nutrient Broth (NB) No. 2 (OXOID) supplemented with 3.6 mM $CaCl_2$ ($NBCaCl_2$), or complete synthetic media, CS2. CS2 synthetic medium was based on the HHWm medium (Toledo-Arana et al. 2005) with the following modifications: 30 mg/l of guanine, 15 mg/l of adenine hemi-sulfate, 8.9 mg/l of $CaCl_2$, 0.08 mg/l of $CuSO_4$, 0.17 mg/l of $ZnSO_4$, 0.12 mg/l of $CoCl_2 \cdot 6H_2O$, 0.12 mg/l of $Na_2MoO_4 \cdot 2H_2O$ (See Table 1 for full composition).

When using synthetic media, cells were collected from overnight cultures by brief centrifugation, and washed with the appropriate medium to be inoculated.

2-3-2. Elimination of N315 prophage from strain N315ex

S. aureus N315 has one prophage in its genome (Kuroda et al. 2001). The N315 prophage is integrated at an *att* site located within the *hlb* β-hemolysin gene,

inactivating the gene and abolishing beta-hemolysis. We used the pMAD system (Arnaud et al. 2004) to precisely excise the phage from the N315ex genome. A set of primers, up-att and down-att, was designed upstream and downstream of *hlb* (See Table 3 and Figure 4C). The target region encompassed by these primers is 46 kbp when phage N315 is present, and 2.4 kbp when the phage is excised. We noted that N315 spontaneously excise the prophage from the genome in a minor fraction of the cells, and the intact *hlb* gene can be amplified by polymerase chain reaction (PCR) (data not shown). The amplified 2.4 kbp *hlb* fragment was cloned into the *Eco*RI - *Bgl*II site of pMAD-tet (Morikawa and Takemura et al. 2012), generating pMAD-tet-att (Figure 4A). The plasmid was then introduced into the strain N315ex and the phage-cured N315ex (N315ex w/oφ) strain was selected as previously described (Arnaud et al. 2004). In addition, beta-hemolysin activity was also monitored. The absence of the *SA1765* and *SA1792* (*ssb*) phage genes in the resultant N315ex w/oφ was confirmed by PCR (primers 1765F and 1765R for SA1765, and 1792ssbR and 1792ssbF for *ssb*) and Southern blot analysis using the 1765F-1765R PCR-generated DNA fragment as a probe (Table 3 and Figure 4B and C).

2-3-3. Construction of *comG* and *comE* mutants from N315ex w/oφ

Deletion/replacement mutants of the *comG* and *comE* regions from N315ex w/oφ strain were constructed by double-crossover homologous recombination as described (Morikawa and Takemura et al. 2012). Briefly, plasmids pMADcomEII (for *comE* mutant) or pMADtetcomGII (for *comG* mutant) were introduced into N315ex w/oφ by electroporation, after the passage through the strain RN4220. Mutants (tetracycline sensitive, β-galactosidase negative) were selected as described.

2-3-4. DNA extraction

The L54a prophage was cured from the COL strain by ultraviolet light treatment as described (McNamara PJ. 2008; Morikawa and Takemura et al. 2012). Plasmid pT181 DNA was then purified from strain COL without phage (COLw/oφ), using the QIAfilter Plasmid Midi kit (QIAGEN). Plasmid pHY300 was also purified from

Escherichia coli (*E. coli*) HST04 *dam*-/*dcm*-, using the QIAfilter Plasmid Midi kit (QIAGEN).

Genomic DNA was extracted by a standard protocol. Cells were collected from 1 ml of overnight culture and suspended with 50 mM Tris- 50 mM NaCl- 1 mM ethylenediaminetetraacetic acid (EDTA). Lysostaphin was added and incubated at 37°C for 20 minutes. After the addition of 200 μl of 0.25 M EDTA-50 mM Tris and 240 μl of 10% sodium dodecyl sulfate (SDS), the sample was incubated at 60°C with occasional inversion and then centrifuged. The supernatant was collected and DNA was harvested by phenol/$CHCl_3$ purification and ethanol precipitation. The DNA suspended in 100 μl of 1/10TE buffer was a treated with 0.5 μl of RNase for 20 min at 37°C. phenol/$CHCl_3$ purification and ethanol precipitation were performed again, the sample was suspended with water and kept at -20°C until use.

2-3-5. Southern hybridization analysis

S. aureus genomic DNA was purified using above procedures. DNA was digested with *Sma*I and separated by electrophoresis on a 1% agarose gel. The separated DNA fragments were transferred to a Hybond-N+ membrane (Amersham Biosciences). Analysis was carried out using the AlPhos Direct labeling kit and the CDP star detection reagent system according to the manufacturer's instructions (Amersham Biosciences). DNA fragments used to prepare the probes were amplified by polymerase chain reaction (PCR) with oligonucleotides sets (SA1765F and SA1765R, see Table 3).

2-3-6. Natural transformation of *S. aureus* cells

Competent *S. aureus* recipient cells were prepared by overnight growth in TSB containing chloramphenicol (12.5 μg/ml) with shaking at 37˚C. Cells were harvested from 500 μl of overnight culture, washed with CS2 medium, resuspended in 10 ml of CS2 medium and grown at 37˚C with shaking. After 8 hours, cells were harvested by centrifugation, and resuspended in 10 ml of fresh CS2 medium. 10 μg of plasmid DNA (pT181 or pHY300 isolated from *E. coli* HST04 *dam*-/*dcm*-) was added to the suspension, and incubation was pursued at 37˚C with shaking for 2 hours. Cells were

mixed into melted BHI-agar pre-cooled to 55˚C together with 5 μg/ml tetracycline and 5 μg/ml erythromycin, and incubated at 37˚C for 2 days. Colonies were tested for their characteristics to confirm that they were *bona fide* recipient cell transformants (e.g. kanamycin resistance and plasmid species). For transformation with chromosomal DNA, 10 μg of N315 genomic DNA was added to the cells. Transformants were selected with 100 μg/ml kanamycin. To check the transfer of genes on the SCC*mec* II, kanamycin gene (*aadD*), *mecA*, and *ccrA* were amplified by PCR using the primer pairs described in Table 3. To detect the entire SCC*mec* II, Long PCR analysis was performed. Three primer sets that cover whole SCC*mec* II sequence were used; Xsaw 325 / mecA-F, ccrA-A-R / 3.0-R, and mecA-R / ccrA-F.

2-3-7. Strain-to-strain plasmid transformation

Cells were grown overnight in TSB with shaking at 37˚C. One hundred μl of donor (COL) and 400 μl of recipient cells (N315 derivative) were mixed and washed with CS2 medium. Cells were resuspended in 10 ml of CS2 medium, and grown at 37˚C for 8~10 hours with shaking. CFU values of N315 derivatives (larger colonies than COL derivatives) after co-cultivation were counted on drug-free BHI-agar plate. Co-cultivated cells were mixed into melted BHI-agar pre-cooled to 55˚C together with 5 μg/ml tetracycline and 5 μg/ml erythromycin, and incubated at 37˚C for 2 days. Colonies were tested for susceptibility to kanamycin to verify the transformants and kanamycin resistant clones were regarded as N315 derivatives.

2-3-8. Pseudo-transformation assays

Phage particle-dependent pseudo-transformation assays were carried out based on the $CaCl_2$ washing method previously described (Pattee and Neveln. 1975) with some modifications. In brief, *S. aureus* cells were grown in TSB medium at 37˚C overnight with shaking (180 rpm, BR-23UM: TAITEC). Cells were recovered by centrifugation and washed once with 0.1 M Tris-malate (pH 7.0). The cells were resuspended in 0.1M Tris-malate (pH 7.0) supplemented with 0.1 M $CaCl_2$. 24 μg of purified N315 genomic DNA was added to 1 ml of the cell suspension, and incubated at room temperature for 40 min. Cells were recovered by centrifugation and suspended in drug-free BHI medium. Following 1 h incubation at 37˚C with shaking, cells were

mixed with molten BHI-agar medium pre-cooled to 55˚C and supplemented with 5 µg/ml erythromycin and poured into plates. After two days incubation at 37 ˚C, colonies were counted and checked for the presence of the *erm* gene by PCR with primers ErmA1 and ErmA2.

2-4 Results

2-4-1. Eliminating phage sequence

The SigH dependent DNA transfer requires both *comG* and *comE* operons, suggesting that this is not so-called "pseudo-transformation" that depends on phage tail components. Since strains COL and N315 have resident prophages (phage L54a and phage N315, respectively), it was necessary to eliminate any possible contribution of phage-components in the experiments of the SigH dependent DNA transfer. Therefore, the prophage was eliminated from N315ex to generate N315ex w/oφ as described in Materials and Methods and Figure 4. The phage elimination was confirmed by southern blotting (Figure 4B).

2-4-2. The SigH mediated transformation is independent of lysogenic phage

Table 4 is the summary of the transformation frequencies (Morikawa and Takemura et al. 2012). N315ex w/oφ h (carrying pRIT-sigH, SigH over-expression plasmid) was transformable with pHY300 plasmid DNA isolated from an *E. coli* dam^-/dcm^- strain (see Materials and Methods), while the vector control strain, N315ex w/oφ v, gave no detectable transformants. This experimental system does not contain any phage particles or phage genes, indicating that SigH-dependent natural competence is phage-independent.

Genomic DNA transformation (SCC*mec* typeII, see methods) was also performed with strain N315ex w/oφ carrying pRIT-sigH. Selected transformants showed *aadD* positive. The PCR analysis of SCC*mec* II of the transformants from phage deletion strains demonstrated that all those Long PCR fragments were positive (Figure 5A and B).

It was also demonstrated that unlike natural genetic competence, phage-dependent pseudo-transformation does not require the competence machinery. In strain RN4220, which has no resident prophages, no transformant was obtained using chromosomal DNA (Table 5). In contrast, when RN4220 lysogenized with phage 11 was used with the $CaCl_2$ washing procedure, transformants were obtained at a very low frequency (between $10^{-8} \sim 10^{-9}$) through phage-mediated "pseudo-transformation" (Table 5). In this same strain, when either the *comG* or *comE*

genes were deleted (strains RKCG and RKCE, respectively; See Materials and Methods and Table 3), there was no significant difference in the number of transformants obtained (Table 5). This indicates that the *comG* and *comE* operons are not required for phage-mediated "pseudo-transformation", in sharp contrast to their essential role in SigH-dependent natural genetic competence as shown in Table 4.

Taken together, above results have demonstrated for the first time that natural genetic competence develops in a SigH-dependent manner in *S. aureus*, allowing transformation by extracellular plasmid or chromosomal DNA as well as HGT between different strains.

2-5. Discussion

Since F. Griffith's pioneering discovery of DNA-mediated transformation in *Streptococcus pneumoniae* (*S. pneumoniae*) (Griffith. 1928), natural genetic competence in low GC % Gram-positive bacteria has been extensively studied in *Bacillus subtilis* (*B. subtilis*) and *S. pneumoniae* and shown to involve the assembly of a complex DNA-binding and uptake machinery, made up of a competence pseudopilus and a DNA translocase (Chen and Dubnau. 2004; Claverys et al. 2009). During the 1970's, although several reports described "pseudo-transformation" of *S. aureus*, this was revealed to be due in fact to contaminating phage tail fragments mediating DNA entry and HGT (Birmingham and Pattee. 1981). Despite many subsequent attempts, natural genetic competence was never successfully demonstrated in *S. aureus* even though sequence analysis readily reveals that its genome carries a practically full repertoire of the required competence gene orthologues, suggesting that specific conditions must exist allowing natural transformation by DNA in *S. aureus*.

Strong similarities and interesting differences exist between the competence pathways of *B. subtilis* and *S. pneumoniae*. Indeed, although in both cases the initial triggering event involves a peptide quorum-sensing two-component signal transduction pathway controlling expression of competence genes encoding the DNA uptake machinery, the steps in between are quite different (Dubnau et al. 1994; Chen and Dubnau. 2004; Claverys et al. 2006). In *Streptococcus* species, competence genes are regulated by ComX (also known as SigX) (Lee and Morrison. 1999), a secondary sigma factor related to staphylococcal SigH (Morikawa et al. 2003) and encoded by

duplicated genes (*comX1* and *comX2*) whose expression is directly controlled by the ComDE two-component system (Lee and Morrison. 1999). Interestingly, in *B. subtilis*, late competence genes are transcribed by the vegetative form of RNA polymerase holoenzyme, $E\sigma^A$, and instead positively controlled by a specific transcription activator, ComK (van Sinderen et al. 1994). Other similarities between the two bacteria include the fact that many additional factors play a part in the production of active ComK or ComX, both of which involve two-component signal transduction networks (Dubnau et al. 1994; Msadek. 1999; Claverys et al. 2006) and the post-transcriptional control of the levels of these two regulatory proteins by the Clp ATP-dependent protease (Msadek et al. 1994; Msadek et al. 1998; Turgay et al. 1998; Msadek 1999; Chastanet et al. 2001; Opdyke et al. 2003; Sung and Morrison. 2005; Claverys et al. 2006).

In *S. aureus*, the situation appears to be more closely related to that of *S. pneumoniae*. Indeed, although a protein bearing some similarities to ComK is present (SA0882), Morikawa et al. have previously identified the SigH secondary sigma factor, analogous to ComX, and shown that it acts specifically to direct transcription of the *comE* and *comG* operons that encode orthologues of the DNA uptake machinery (Morikawa et al. 2003).

Whereas in *S. pneumoniae* all of the cells become competent for a short period in time, in *B. subtilis* only a maximum of 10% of the cell population achieves competence (Claverys et al. 2006), a fact that has been attributed to the positive autoregulatory feedback loop controlling *comK* expression, generating a heterogeneous bistable response in the cell population (Maamar and Dubnau. 2005; Smits et al. 2005; Maamar et al. 2007). However, it is important to recall that natural undomesticated strains of *B. subtilis* are in fact considered to be non-competent (Nijland et al. 2010), and that transformation of *B. subtilis* at levels of 10% could be demonstrated only for a few strains isolated following extensive UV and X-ray mutagenesis (Burkholder and Giles. 1947; Spizizen. 1958), with the highly transformable 168 strain then chosen for most studies (Anagnostopoulos and Spizizen 1961). *B. subtilis* strains derived from 168 rapidly became “domesticated” once exposed to the accelerated lifestyle imposed in the laboratory, accumulating multiple

mutations affecting competence development and biofilm formation (Earl et al. 2007; Zeigler et al. 2008; McLoon et al. 2011).

Thus, the situation for *Staphylococcus aureus* appears highly reminiscent of that of undomesticated *B. subtilis*, with a cryptic DNA uptake apparatus presumably allowing only a very low level of natural transformation in its natural habitat, with the possibility that specific conditions may be required for competence development. Limiting the number of competent recipient cells in a population would be important to sustain genome integrity, minimizing risks and maximizing evolutionary gain by allowing only a fraction of the cells to access genetic variability. Among the many barriers to uptake of foreign DNA, restriction-modification systems are known to play an important role (Waldron and Lindsay. 2006; Veiga and Pinho. 2009; Corvaglia et al. 2010), and tight control of competence gene expression is also required to limit potentially detrimental HGT with other species (Claverys et al. 2006). In this respect it is tempting to speculate, given the observed population heterogeneity with respect to SigH activity, that natural competence in *S. aureus* has evolved as a bet hedging strategy (Veening et al. 2008), with most of the cells protected against the dangers of HGT, while a fraction are able to increase genetic variability through natural genetic competence.

As ComK and SigX (ComX) are the end products of the regulatory cascades controlling competence development in *B. subtilis* and *S. pneumoniae*, several attempts have been made to overproduce these proteins in non-competent bacteria in order to obtain genetic transformation. In *Streptococcus pyogenes* (*S. pyogenes*), which is not known to become competent, SigX has been shown to control expression of *femB* and *cinA* (Opdyke et al. 2003), as well as competence gene orthologues (Woodbury et al. 2006). Likewise, overproduction of SigX in *Lactococcus lactis* (*L. lactis*) also led to increased expression of competence gene orthologues (Wydau et al. 2006). The recent discovery among *Streptococcus* species of a second quorum-sensing pathway allowing activation of *sigX* expression (Fontaine et al. 2010; Mashburn-Warren et al. 2010; Okinaga et al. 2010) has led to the suggestion that members of the pyogenic streptococci group may in fact be able to develop competence under specific conditions (Havarstein, 2010; Mashburn-Warren et al. 2010).

In a similar approach, overproduction of the ComK transcription activator from *B. subtilis* was used to induce competence in otherwise non-competent bacteria. This approach was successful both in undomesticated strains of *B. subtilis* (Nijland et al. 2010) as well as in *Bacillus cereus*, previously considered to be non-competent (Mironczuk et al. 2008; Kovacs et al. 2009). Interestingly, *B. cereus* carries two copies of the *comK* gene, reminiscent of the *comX* situation in *S. pneumoniae*, although ComK1 and ComK2 appear to play different roles (Mironczuk et al. 2011).

In *S. aureus*, *sigH* has also been reported to direct transcription of phage integrase genes to stabilize the lysogenic state (Tao et al. 2010). This study has shown that SigH is required for competence development in a minor fraction of the cell population. SigH may also protect the subpopulation from phage-induced lytic death, allowing survivors to utilize dead cell materials, including DNA, with a higher probability of acquiring new genes through HGT.

The demonstration that natural genetic transformation of *S. aureus* cells occurs in a SigH-dependent manner helps provide an explanation for the notorious acquisition of antibiotic resistance genes by this major pathogen. Indeed, even at very low natural competence levels, the selective pressures would ensure the survival and rapid spread of strains acquiring antibiotic resistance genes, as currently observed for methicillin-resistant *S. aureus* (MRSA) strains (Chambers and Deleo. 2009).

Importantly, a large chromosomal region conferring methicillin resistance (SCC*mec* type II) could be transferred by transformation. However, it should be noted that N315ex cell was used as the recipient, which had lost the SCC*mec* element, resulting in a methicillin-sensitive *S. aureus* (MSSA) phenotype. The transfer of SCC*mec* into naive MSSA strains needs to be tested, but will require optimization of the transformation protocol.

Chapter 3

Sodium Polyanethol Sulfonate Modulates Natural Transformation of SigH-Expressing *Staphylococcus aureus*

3-1. Abstract

Expression of genes required for natural genetic competence in *Staphylococcus aureus* (*S. aureus*) was controlled by an alternative transcription sigma factor, SigH. However, even in the SigH-expressing cells, the DNA transformation efficiency varied depending on culture conditions. This chapter shows that cells grown in the competence-inducing medium (CS2 medium) exhibits enlarged morphology with disintegrated cell walls. Notably, an autolysis inhibitor, Sodium Polyanethol Sulfonate (SPS), facilitated transformation in CS2 medium in a dose-dependent manner, suggesting the involvement of the cell wall metabolism in transformation. The transformation efficiency was not improved by physical or enzymatic damage on the cell walls.

3-2. Introduction

Subpopulations of *S. aureus* can develop the competence for natural DNA transformation under the control of the alternative sigma factor, SigH (See Morikawa and Takemura et al. 2012. and Chapter 2). In addition to SigH expression (and SigH-dependent expression of the *comE* and *comG* operon genes encoding the DNA incorporation machinery), environmental factors are thought to be required for natural transformation, because the transformation frequency of SigH-expressing cells is variable depending on the culture conditions. Cell wall-affecting antibiotics were also found to affect the transformation in SigH-expressing cells (Nguyen et al. 2016). Bacitracin, which interferes with peptidoglycan synthesis, increased the transformation frequency at low concentrations and decreased the frequency at higher concentrations. Other cell wall-targeting antibiotics, vancomycin and fosfomycin, increased the transformation frequencies. In contrast, an antibiotic interfering protein synthesis, streptomycin, and antibiotics targeting DNA gyrase such as ciprofloxacin and norfloxacin showed no significant effect. Since bacitracin, vancomycin and fosfomycin are cell wall-affecting antibiotics, the cell wall integrity or metabolism could be important for transformation.

This chapter shows that the autolytic enzyme inhibitor, Sodium Polyanethol Sulfonate (SPS), affects the transformation efficiency of SigH-expressing *S. aureus,* supporting the idea that cell wall metabolism is an important factor in the modulation of transformation.

3-3. Material and Methods

3-3-1. Bacterial Strains

The *S. aureus* strains used in this study are listed in Table 6. The strain N315ex w/oφ h was used in most experiments. In this strain, the prophage was eliminated to exclude the possibility of "pseudo-competence" DNA transfer with the help of phage components, which is distinct from real competence (Chapter 2). SigH is expressed by a plasmid, pRIT-sigH (Morikawa and Takemura et al. 2012.).

3-3-2. Natural Transformation Assay

Transformation assay was carried out as described in Chapter 2 with some modifications. Tryptic soy broth (TSB; Becton Dickinson Company), brain heart infusion broth (BHI; Becton Dickinson Company), nutrient broth (NB) (Oxoid) supplemented with 70 μM $CaCl_2$ ($NBCaCl_2$), and the complete synthetic medium, CS2 (composition is available in Table 1), were tested for the efficiency of transformation in N315ex w/oφ h cells. Transformation protocol was the same for all the media tested. Glycerol stocks of *S. aureus* were inoculated in 5 ml of tryptic soy broth with 12.5 μg/ ml chloramphenicol (TSBcm) and grown overnight at 37 ° C with shaking at 180 rpm. Cells were collected by centrifugation and suspended into the appropriate medium to be used (1:20 dilution in 10 ml medium), and grown for 8 h. Sodium polyanethol sulfonate (SPS) (Sigma) was added prior to the 8-hour growth. Medium was replaced with fresh medium, and 10 μg of purified plasmid, pT181 from *S. aureus* COL, or pHY300 from *E. coli* HST04 *dam−/dcm−,* was added. Following 2.5-h incubation at 37 °C with shaking, transformants were selected in BHI-agar medium supplemented with 5 μg/ml tetracycline.

Transformants were tested for the presence of plasmid and for *tetR* by colony polymerase chain reaction (PCR). In line with our previous experiences, no spontaneous *tetR* mutants were detected throughout the study. Transformation frequency was calculated as the ratio of total number of transformants to total colony forming unit (cfu) after the 2.5-h incubation with DNA. Cfus were counted on TSB-agar (TSA) plates.

3-3-3. Electron Microscopy

Overnight cultures of bacteria (N315 h, N315 v, COL h, COL) in TSBcm or TSB were inoculated into drug-free TSB or CS2 to a final optical density (OD_{600}) of 0.2. After 8 h culture, cells were harvested by centrifugation. After a complete wash in ice-cold phosphate buffered saline (PBS), cells were fixed with 2% glutaraldehyde in PBS followed by post-fixation with 1% OsO_4, dehydrated, embedded, and thin sectioned by conventional method described elsewhere (Anderson et al. 2004). The specimens were observed using a JEOL JEM-1400 electron microscope. The cell surface roughness was evaluated from electron microscopy images by measuring the

ratio of the length of the cell surface to the corresponding linear distance (i.e., the ratio 1.0 means completely smooth, and larger value means rough morphology).

3-3-4. Whole Cell Autolysis Assay

Whole cell autolysis assay was performed as described by Mani et al. (Mani et al. 1993). Cells from the overnight TSBcm culture of N315ex w/oφ h were inoculated with 1:20 dilution in 10 ml TSB or CS2 with or without 0.1% SPS. For CS2, cells were washed once with fresh CS2 prior to inoculation. After 8 h (in CS2) or 5 h (in TSB), cells were harvested by centrifugation. Cells were washed twice with 10 ml of ice- cold water and resuspended in 10 ml of 0.05 M Tris‑HCl buffer (pH 7.2) containing 0.05% (v/v) Triton X-100. Two hundred microliters of the cell suspension was distributed into a 96-well plate, and then incubated at 30 ˚C with shaking at 180 rpm. The changes in OD_{600} were measured at 30 min intervals using the EnSpire® Multimode plate reader (PerkinElmer®).

3-3-5. Zymographic Analysis

The supernatant from 8-h culture of N315ex w/oφ h in CS2 or 5-h culture in TSB was recovered by centrifugation at 6000×g for 15 min at 4 ˚C, filtered through 0.22-μm cellulose acetate membrane filter (Advantec®, Toyo Roshi Ltd.), and concentrated 10-fold using Ultracel® 10K Centrifugal filter unit (Merck Millipore). Sodium dodecyl sulfate (SDS) extracts were prepared as previously described (Sugai et al. 1990). Protein concentrations were determined by the Bradford assay (Bio- Rad Laboratories). Twenty micrograms of total proteins were analyzed by zymogram as described by Qoronfleh and Wilkinson using *Micrococcus luteus* (Sigma) as a substrate (Qoronfleh and Wilkinson. 1986).

3-3-6. Mechanical and Enzymatic Disruption of Cell Walls

Fastprep® (MP Biomedicals) was used to physically disrupt bacterial cell walls. A TSBcm overnight culture of N315ex w/oφ h was diluted 20-fold with fresh TSBcm (total 10 ml) and cultured for 8 h. Cells were harvested, suspended in 0.4 ml TSB, and transferred to 2-ml tube containing 0.1- mm silica beads (MP Biomedicals). The tube

was shaken in Fastprep® at 4 m/s for 10, 20, and 30 s, once or twice, at 4 ˚C. After beating, cells were collected by centrifugation (10,000 rpm, 10 min) and cell-bead mixture was resuspended in 1 ml TSB. The cell suspension was transferred to a new tube, and 10 μg of purified plasmid pHY300 was added to test the transformation frequency.

For lysostaphin treatment, cells grown in 5 ml TSBcm were harvested at the mid-log phase. Cells were then suspended in 10 ml TSB medium containing lysostaphin at different concentrations and incubated for 5 h at 37 °C with shaking. After treatment, cells were washed and mixed with purified pHY300 for transformation.

3-4. Result

3-4-1. CS2 Medium is Important for the Transformation of SigH−Expressing Cells

In the transformation protocol reported previously, SigH-expressing cells (N315ex w/oφ h) were growth in CS2 medium (Table 1). The medium dependency was confirmed here and summarized in Table 7. Transformation was undetectable (less than 10^{-11}) in TSB, BHI, and NBCaCl$_2$ (Nutrient broth supplemented with 70 μM CaCl$_2$) but it reached 10^{-9} order in CS2 medium, when pT181 plasmid purified from *S. aureus*. COL was used as the donor DNA. A shuttle vector, pHY300, purified from *Escherichia coli* (*E. coli*), was also tested and observed the similar dependency on the CS2 medium (Table 7).

3-4-2. *S. aureus* Cells in CS2 Medium Exhibit Disintegrated Cell Walls

N315 derivative strains including N315ex w/oφ h tend to generate cell debris in CS2 medium, but not in other media, irrespective of the presence of prophage and the SigH expressing plasmid. This was not the case in COL and COL h strains, of which transformation was undetectable in the same CS2 protocol. The strains N315h (Figure 6A, B), N315v (Figure 6C), N315 (Figure 7A), and COL (Figure 7B) were observed by transmission electron microscopy. N315 overexpressing SigH (N315 h) grown in CS2 medium (Figure 6B), but not in TSB (Figure 6A), exhibited irregular morphology and enlarged cell size with partly disturbed cell wall. Such morphological features were unique in N315 background (Figure 7A), not observed in COL (Figure 7B), and are not attributed to the artificial expression of SigH, since cells carrying the vector control (N315 v) showed similar changes when grown in CS2 medium (Figure 6C).

3-4-3. Inhibitor of Murein Hydrolases Increases Transformation in CS2 Medium

The autolytic rate of the cells grown in CS2 medium was higher than those grown in TSB medium (Figure 8A), and zymogram analyses indicated that autolytic enzymes in CS2 medium are at a comparable level with TSB medium (Figure 8B). The effect of a murein hydrolase inhibitor, SPS (Wecke et al. 1986) was tested. The inhibitory effect of SPS on the autolysis of the CS2-grown cells was confirmed as shown in

Figure 6A. Zymogram indicated that the autolysin Atl (51 and 62 kDa) and LytM (35 kDa) were strongly reduced by SPS in the SDS extract, and it was undetectable in culture supernatant (Figure 8B). Strikingly, SPS increased transformation frequency in a dose- dependent manner in CS2 medium (Figure 8C), but not in TSB medium (Figure 8D). The addition of 0.1% SPS in CS2 medium resulted in 10-fold increase in the transformation frequency ($p < 0.01$) (Figure 8C).

To exclude the possibility that SPS somehow induced plasmid incorporation without competence machinery, SigH-active cells lacking the *comE* or *comG* operon (N315ex w/oφ ΔcomE h, N315ex w/oφ ΔcomG h) were tested. No transformant was detected from these strains. Thus, SPS can affect natural transformation in CS2 medium.

3-4-4. Physical Damage on Cell Wall Does Not Facilitate Transformation of SigH Expressing *S. aureus* in TSB

To address whether the increase in transformation of SigH-expressing cells would be simply attributed to the physically disturbed cell wall, the effect of physical disruption by silica beads on the transformation of cells grown in TSB was tested. Cfu was measured at different time points of bead beating. Beating twice for 10, 20, and 30 sec (10×2, 20×2, and 30×2) resulted in 30, 45, and 60% reduction in cfu. None of the treatments improved the transformation efficiency (Figure 9A). In addition, the effect of lysostaphin, an enzyme that cleaves *S. aureus* cell wall (Kumar. 2008) was also tested. Any increment in the transformation frequency by lysostaphin treatment up to the concentration that reduced the cfu by half (0.1 μg/ml) was not observed. Higher concentrations of lysostaphin resulted in undetectable transformants due to cell death (Figure 9B). Thus, external physical damage on the cell wall does not simply facilitate the transformation.

3-5. Discussion

Regulatory mechanisms and signals for the development of natural competence are diverse among species (Johnston et al. 2014). In 2012, *S. aureus* natural transformation is dependent on SigH (Chapter 2). The transcription factor ComK was also found to enhance the expression of the SigH regulon (Fagerlund et al. 2014). However, the regulation of competence development and the following transformation are still largely unknown. This chapter aimed to gain insight into the regulation of staphylococcal competence, of which frequency is still low in laboratory settings. Although the SigH-expressing strain had to be used to evaluate the transformation frequency, the distinct transformation efficiencies of SigH-expressing cells in different culture media was clarified, and the effect of SPS on the transformation was firstly described.

Transformation in SigH-expressing cells is facilitated in CS2 medium compared with other complex rich media (BHI, TSB, LB). The growth rate decreases and the cell size increases in CS2 medium (Figure 10 and 11). SigH in normal cell (N315ex) is expressed in up to 10% subpopulation in CS2 (Morikawa and Takemura et al. 2012), but the increase of the cell size is observed for almost all N315ex cells. In addition, the cell size increase was at comparable level between N315ex, N315ex h, and N315ex Δ sigH (Figure 11). Therefore, such a morphological change is not under the control of SigH. The addition of 0.1% SPS did not change the cell size in CS2 and TSB (Figure 11), suggesting that impaired autolytic activity is not the sole reason for the cell size difference.

The slow growth during competence, and delayed cell division is also observed in *Bacillus subtilis,* where ComGA and Maf interfere with rRNA synthesis and cell division, respectively (Briley et al. 2011; Hahn et al. 2015). It is thought that the arrest of growth could permit cells to express competence machinery genes and internalize exogenous DNA. It could also permit the repairing of the bacterial genome after the recombination during transformation (Briley et al. 2011; Hahn et al. 2015). Whether the enlarged cell size is a prerequisite for the staphylococcal transformation is elusive, but in general, it is conceivable that optimal growth conditions where cells divide at high rate are not suitable for competence development.

SPS affects cell wall turnover due to the inhibition of the cross wall separation (Wecke et al. 1986). Although the direct mechanism by which SPS inhibits the release of autolytic enzymes is not well understood yet, it was suggested that SPS binds to wall teichoic acids and changes the molecular conformation of the cell wall matrix resulting in the inhibition of not only the septum but also the peripheral wall autolytic enzymes. This binding might cause a considerable shift in the net charge of the cell wall since SPS is negatively charged (Wecke et al. 1986). Therefore, this study will not conclude that the effect of SPS on the transformation is through the inhibition of autolytic enzymes: it might be due to such a drastic change of the physical characteristics of the cell walls or other unknown reasons. Nonetheless, taken together with our previous report that some cell wall-affecting antibiotics modulate transformation of SigH-expressing cells, it is likely that cell wall metabolism plays an important role in natural genetic competence in *S. aureus*. The importance of cell wall metabolism in transformation is also recognized in other Gram-positive species including *B. subtilis, S. pneumoniae,* and other streptococci (Bayles. 2007; Ranhand. 1973).

The physical disruption of the cells did not increase the transformation efficiency in TSB medium. This suggests that the increase in transformation frequencies of SigH-expressing cells cannot simply be attributed to the damages in the cell wall alone. In this context, it is valuable to note that the ComC disulfide bond is necessary for the maturation of ComG pseudopilin (van der Kooi-Pol et al. 2012). A study in *S. pneumoniae* reported that competence accessories (EndA nuclease and the DNA receptor ComEA) are recruited near the septum and the DNA uptake could occur at this position (Berge et al. 2013). Thus, the present data are consistent with the idea that the DNA uptake would be finely controlled through such multiple processes.

In conclusion, this study added new information that SPS can facilitate natural transformation in SigH-expressing *S. aureus*. Together with the previous report that cell wall-affecting antibiotics can affect transformation (Thi le et al. 2016), this study supports the idea that cell wall metabolism plays an important role in the DNA incorporation process by the competence machinery expressed by SigH.

Chapter 4

Future directions

Relevance of transformation in *S. aureus*

In bacterial kingdom, horizontal gene transfer (HGT) is the strong driving force for species evolution, and ubiquitous strategy to sustain genetic diversity and dynamics. Among the three major mechanisms of HGT, natural genetic competence alone is the ability of recipient-side cells. The development of natural competence is highly risky decision for bacteria, because incoming genetic information, once it is stably acquired, is often unnecessary burden or even toxic. Therefore, each bacterial species evolved a series of regulation in terms of extracellular DNA incorporation. A series of species-specific regulation for the competence gene expression has been described, e.g. absolute dependency on the catabolite regulator cyclic AMP receptor protein (CRP) in *Haemophilus influenzae* (*H. influenzae*) (Redfield et al. 2005), requirement of low level CO_2 condition in *Helicobacter pylori* (*H. pylori*) (Moore et al. 2014), chitin in *Vibrio cholerae* (*V. cholerae*) (Meibom et al. 2005.), or high cell density (quorum-sensing) in some Gram positive bacteria. Furthermore, some bacteria have established systems that selectively incorporate the DNA from its own species; *H. influenzae* and *Neisseria gonorrhoeae* (*N. gonorrhoeae*) have receptors for the specific "DNA uptake sequence", while *H. pylori* utilizes extracellular restriction-modification system (Seitz and Blokesch. 2013). This study firstly found that *S. aureus* natural competence is regulated by the cryptic sigma factor that expresses in minor cell population, thereby protecting majority of cells from the risky trial to incorporate extracellular DNA. It is likely that *S. aureus* might have evolved to utilize natural competence as a kind of hedge-betting strategy, rather than nutrient acquisition, or DNA repair mechanism, which must be clarified in future studies.

Role of transformation in transfer of large DNA segments.

The bacteriophages in staphylococci can transduce up to 45 kbp DNA, suggesting that most types of mobile genetic elements could be transduced by bacteriophages. In other bacteria such as *Bacillus* and *Pseudomonas*, large phages with DNA contents of

over 200 kbp called as ‘‘jumbo bacteriophages’’ have been reported (Drulis-Kawa et al. 2014). Similarly, environmental giant phage that can accommodate up to ca. 270 kbp DNA was fond to transduce DNA to *S. aureus* (Uchiyama et al. 2014).

In spite of these evidences, transduction of SCC (size ranges from a few kbp to 60 kbp) has long been difficult and only a few reports describes type IV and I SCC transduction (Ito et al. 2001; Scharn. 2013). Moreover, there are evidences of horizontal transfer of much larger DNA segments that cannot be accommodated even in giant phages: In certain lineage termed ST239 (distinguished based on multilocus sequence typing (MLST; Enright et al. 2000)), the genome possesses 240 ~ 550 kbp DNA insertion which is considered to be of evolutionary distinct lineage ST30 (Robinson and Enright. 2004). In this context, the natural transformation found in this study is the potential pathway for these large DNA to be transferred (Figure 12). However, the transformation frequency described is extremely low. Further efforts is necessary to explore for unknown conditions for efficient natural transformation, and to test if it is responsible for large DNA transfer including each type of SCC.

The contribution of this study to the world wide problem in the antibiotic resistance

As World Health Organization (WHO) has pointed out, the emergence of antibiotics-resistant bacteria is a global assignment. Even though human race has developed antibiotics, antibiotics-resistant bacteria have emerged one after another, telling us how difficult to control the emergence of resistant bacteria. Penicillin-resistant *Staphylococcus* was reported three years before the introduction of penicillin into the market. Two years after methicillin developed in 1960, methicillin-resistance *Staphylococcus* was reported. While some resistances are due to gene mutation (such as resistance against rifampicin and quinolones), many resistances are due to HGT of resistance genes on mobile genetic elements (such as against β-lactam, chloramphenicol, tetracycline, glycopeptide).

The incidence of the antimicrobial resistance is increasing year by year. In the case of *Staphylococcus* spp., a significant increase in the isolation of nosocomial Methicillin resistant *Staphylococcus aureus* (MRSA) strains has been observed; the

percentage increased from 2.4% in 1973 to 35% in 1996 in the United States and from 1.7% in 1990 to 8.4% in 1995 in Germany (Martins et al. 2007; Witte. 1999). The incidence of methicillin-resistant *Staphylococcus epidermidis* (*S. epidermidis*) in Finland also increased from 28% in 1983 to 77% in 1994. According to WHO, mortality of patients infected with MRSA is estimated to be 64% higher than that of patients infected with non-resistant *S. aureus*, and around ten thousand people die due to MRSA infections in one year. In 2007, it was found that, in certain US regions, the death rate due to MRSA was higher than the death rates due to AIDS (Klevens et al. 2007). Furthermore, recent MRSA isolates are resistant to many antibiotics besides β-lactam antibiotics (Hiramatsu et al. 1998). Such multi-drug resistance bacteria called as "superbugs" are now spreading, being the fear of returning to the "pre-antibiotics" era. In addition to the antimicrobial resistance (AMR) action plans from WHO (World Health Organization, 2015), and each region/ country (United States, 2015; Japan, 2016), research on the evolutionary ability of pathogens might give us new ways to cope with the AMR issue. Through the present study, the HGT by the natural competence was understood as the tightly regulated process, and indeed, environmental factors were found to regulate the HGT efficiency. Further study is necessary to get the hint for us to live well together with this evolutionary successful opportunistic human pathogen.

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List of publications

(1) Original Papers

1) Kazuya Morikawa, Aya J. Takemura, Yumiko Inose, Melody Tsai, Le Thuy Nguyen Thi, Toshiko Ohta, Tarek Msadek. "Expression of a cryptic secondary sigma factor gene unveils natural competence for DNA transformation in *Staphylococcus aureus.*" *PLoS Pathogens*. Nov 1st. 2012. Vol. 8, e1003003.
The chapter 2 refers to this article.

2) Kouhei Mizuno, Mamiko Mizuno, Mio Yamauchi, Aya J. Takemura, Veronica Medrano Romero, Kazuya Morikawa. "Adjacent-possible ecological niche: growth of *Lactobacillus* species co-cultured with *Escherichia coli* in a synthetic minimal medium." *Scientific Reports*. Oct 16th. 2017. 7(1):12880. doi: 10.1038/s41598-017-12894-3.

3) Le Thuy Nguyen Thi[#] Aya J. Takemura[#], Ryosuke L. Ohniwa, Shinji Saito, Kazuya Morikawa. "Sodium Polyanethol Sulfonate modulates natural transformation of SigH-expressing *Staphylococcus aureus*." *Current Microbiology*. Dec 5th. 2017. doi: 10.1007/s00284-017-1409-5. [#]These authors contributed equally to this work.
The chapter 3 refers to this article.

(2) Others

1) Aya J Takemura, Yuri Ushijima, Le Thuy Nguyen Thi, and Kazuya Morikawa. "Classical *rsbU*- strains of *S. aureus* exhibit better growth in synthetic nasal medium." *Microbes in the spotlight: recent progress in the understanding of beneficial and harmful microorganisms. BrownWalker Press*. Ed: A. Méndez-Vilas 464-467. July. ISBN-10: 1627346120 ISBN-13: 9781627346122. 2016. *Proceedings paper.*

List of presentation

(1) Presentation in international conferences

-Posters

1) Aya J. Takemura, Kazuya Morikawa. "Minor cell specific expression of staphylococcal *sigH* involves translational control by inverted repeat sequence in 5'-untransrated region." 6th Tsukuba Medical Science Research Meeting/ The 2nd Leading Graduate Schools International Conference. Japan. 2011 Nov. No.

2) Aya J. Takemura, Kazuya Morikawa, Le Thuy Nguyen Thi, Melody Tsai, Yumiko Inose, Toshiko Ohta, Tarek Msadek. "Expression of a Cryptic Sigma Factor Unveils Natural Competence for DNA Transformation in *S. aureus.*" 7th international Conference on Gram-Positive Microorganisms/ 17th International Conference on Bacilli. Italy. 2013 Jun.

3) Le Thuy Nguyen Thi, Aya J. Takemura, Yumiko Inose, Melody Tsai, Toshiko Ohta, Tarek Msadek, Kazuya Morikawa. "First evidences of genetic transformation via natural competence in *Staphylococcus aureus*." International Congress on Bacteriology and Infectious Diseases Baltimore. USA. 2013 Nov.

4) Aya J Takemura, Kazuya Morikawa. "A part of Agr-deficient 'stealth' Staphylococcus aureus can regain its virulence during phagocytosis." Tsukuba Global Science Week 2015. Japan. 2015 Sep.

5) Aya J. Takemura, Kazuya Morikawa. "Reversible mutations in agr locus in Staphylococcus aureus." VI International Conference on Environmental, Industrial and Applied Microbiology. Spain. 2015 Oct.

6) Vishal Gor, Aya Takemura, Lisa Maudsdotter, Kazuya Morikawa. "Survival of *Staphylococcus aureus* under dry stress conditions: involvement of expression in sub-population genes." Japan. 2017 Sep.

(2) Presentation in domestic conferences

-Oral

1) Kazuya Morikawa, Vishal Samir Gor, Aya J. Takemura, Lisa Maudsdotter. "黄色ブドウ球菌が乾燥下で生き抜くメカニズム: 限定的発現遺伝子の関与の可

能性　シンポジウム “Sleeping Microbes:眠れる微生物の秘めたる力” Sendai. 2017 Mar.

-Posters

1) Vishal Samir Gor, Aya Takemura, Kazuya Morikawa. “Two *esp* (expression in minor subpopulation) genes confer dry stress resistance to Staphylococcus aureus.” Sendai. 2017 Mar.

2) Aya Takemura, Le Thuy Nguyen Thi, Toshiko Ohta, Kazuya Morikawa. “Expression of a Cryptic Sigma Factor Unveils Natural Competence for DNA Transformation in *S. aureus.”* Makuhari. 2013 Mar.

3) Aya Takemura, Ryosuke Ohniwa, Shinji Saito, Kazuya Morikawa. “Minor cell specific expression of staphylococcal *sigH* involves translational control by inverted repeat sequence in 5’-untransrated region.” Yokohama. 2011 Dec.

4) Aya Takemura, Kazuya Morikawa. “Exploring for new bet-hedging strategy in Staphylococcus aureus.” Hunabori. 2014 Mar.

5) Aya Takemura, Kazuya Morikawa. “*S. aureus* expresses a number of genes with unknown function in the subpopulations.” 88th. Gifu. 2015 Mar.

(3) Others

-Prize

1) Group J (Aya Takemura, Hiroshi Mieno, Taiki Naitou, Yuya Yamashita, Yuichiro Watanabe, Marcin Pawel Jarzebski, Nagato Sakaki, Jouchi), “Student excellent presentation award.” Program for Leading Graduate School Forum 2012. Tokyo. 2013 May.

2) Aya Takemura. “The 7th Tsukuba Medical Science Research Meeting/ The 3nd Leading Graduate Schools International Conference, Good Discussion Award” Japan. 2012 Nov.

3) 4-d (Aya Takemura, Kenji Irie, Takamichi Matsusako, Takashi Tsuchiya, Syu Kanno, Naoko Kawate, Dai Moriwaki. See-D contest 2012. Double “Senior prize”. Japan, 2012 Dec.

4) Aya Takemura. “Tsukuba Global Science Week 2015, Outstanding Poster Award”, Japan. 2015 Sep.

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Figures and Tables

Chapter 1
Figures and Tables

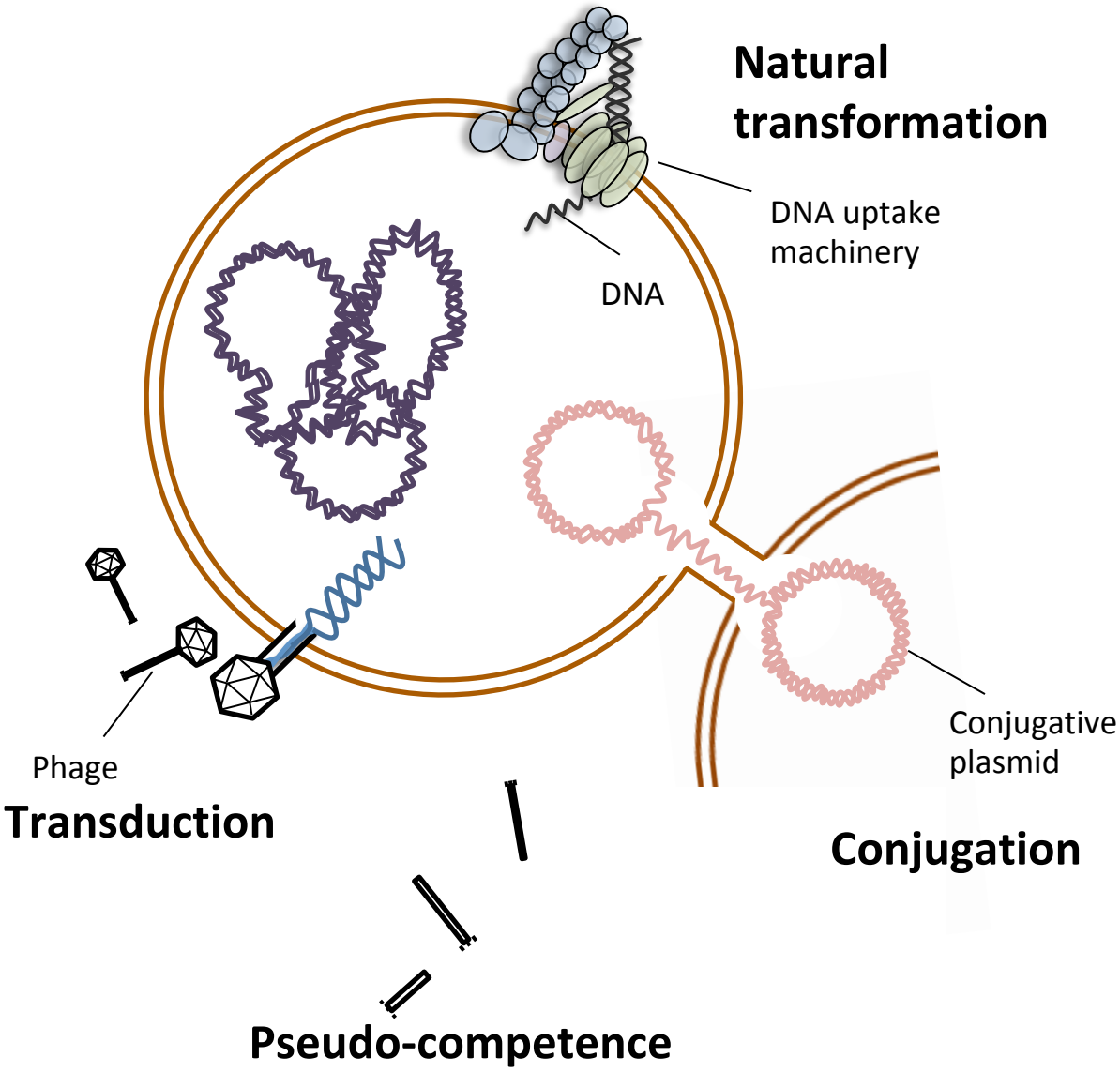

Figure 1.

Horizontal gene transfer in *S. aureus*.
Natural transformation is through the DNA uptake machinery (termed competence machinery) expressed at the cell surface. Conjugation is cell-to-cell DNA transfer mediated by conjugative apparatus. Transduction is mediated by phages. In addition, distinct phage-dependent gene transfer systems are also known (pseudo-competence etc.).

	B. subtilis	*S. pneumonia*	*S. aureus*	% aa identity (*S. aureus* vs *B. subtilis*)
Prepilin peptidase	*comC*	*SP1808*	*SA1486*	27.4
DNA receptor, transprot (see text for details)	*comGA*	*SP2053(cglA)*	*SA1374*	36.7
	comGB	*SP2052(cglB)*	*SA1373*	21
	comGC	*SP2051(cglC)*	*SA1372*	41.4
	comGD	*SP2050(cglD)*	*SA1371*	27.8
	comGE	*SP2049(cglE)*	*SA1370*	18.5
	comGF	*SP2048(cglF?)*	*SA1369*	21.3
	comGG	*SP2047(cglG?)*	×	
	comEA	*SP0954(celA)*	*SA1418*	37.8
	comEB	×	*SA1417*	67.5
	comEC	*SP0955(celB)*	*SA1416*	31
	comFA	*SP2208(cflA)*	*SA0705*	39.6
	comFB	×	×	
	comFC	*SP2207(cflB)*	*SA0706*	29.9
Single-strand DNA binding protein	*ssb*	*SP1540*	*SA1792*	63.6
DNA processing	*smf*	*SP1266(dprA)*	*SA1092*	38.4
Competence protein	*yjbF*	*SP0978(coiA)*	*SA0858*	23.2
Competence damage inducible protein	*cinA*	*cinA(SP1941)*	*cinA*	43.8
Recombination	*recA*	*recA(SP1940)*	*recA*	74
Mismach repair	*mutS*	*mutS*	*mutS1137*	54.8
Nuclease	*nucA*	*SA1964(endA)*	*?*	
Alanine recemase	*dal*	*SP1698*	*alr*	40
UDP-N-acetylglucosamine 1-carboxyviny transferase	*murA*	*SP1081*	*murA(1902)*	67.9

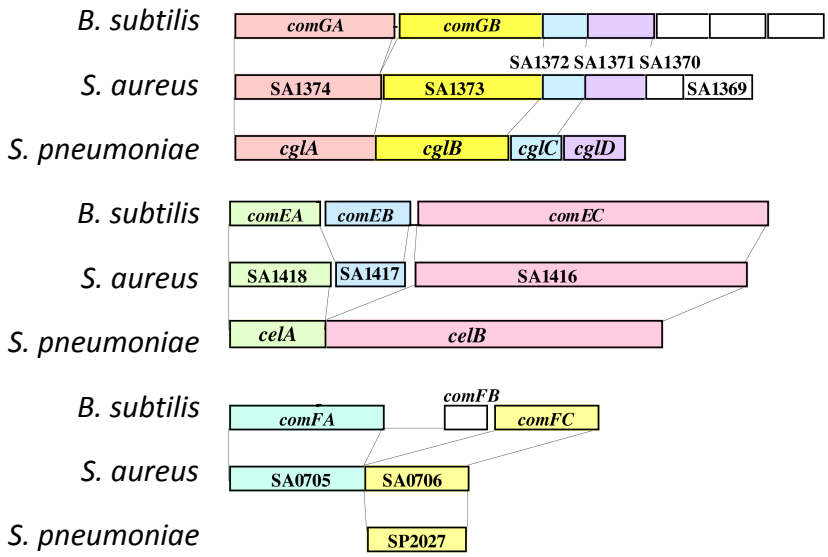

Figure 2.

The orthologous of competence related genes.

The top table shows the presence or absence in *S. aureus* of competence related orthologous genes of *B. subtilis* or *S. pneumoniae*. Percent identities were calculated by the Lasergene software package after pairwise alignment. The figure shows the similar gene organization of the *comG*, *comE*, and *comF* operons in *B. subtilis*, *S. aureus*, and *S. pneumoniae*.

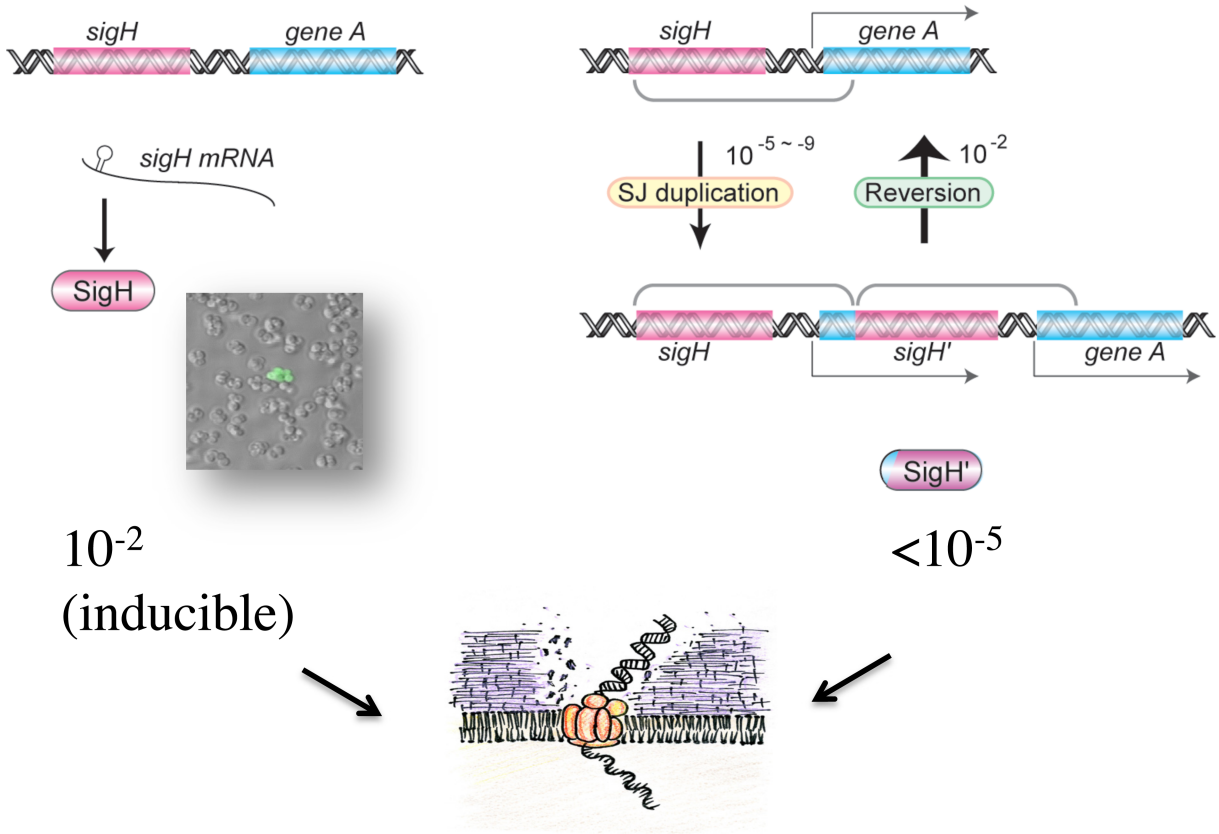

Figure 3.

Competence development in *S. aureus* involves two distinct mechanisms. (right) A rare SJ-duplication mechanism generates a chimeric *sigH* gene, and SigH is produced as a fusion protein. The duplication is cured at a high frequency. (left) Under the specific culture conditions, SigH was expressed at a frequency of ca. 10^{-2} through a post-transcriptional regulatory mechanism. The inverted repeat sequence upstream of the translation initiation site prevents SigH expression, likely forming a secondary structure trapping the ribosome binding site, and/or serving as a post-transcriptional regulatory target, restricting SigH expression to a minor fraction of the cell population.
(bottom) In either case, SigH active cells express genes for DNA-binding and uptake machinery.

	stock conc.	final conc.	1000 ml
Sterile MilliQ water			710 ml
Solution A			100 ml
Na_2HPO_4	71.4 g/L	7.1 g/L	
KH_2PO_4	30 g/L	3 g/L	
$(NH_4)_2SO_4$	20 g/L	2 g/L	
Solution B-1			10 ml
$MgSO_4 \cdot 7H_2O$	5 g/L	50 mg/L	
$MnSO_4 \cdot 5H_2O$	0.54 g/L	5.4 mg/L	
Solution B-2			10 ml
$FeSO_4 \cdot 7H_2O$	0.28 g/L	2.8 mg/L	
Solution C			25 ml
Glucose	40 %(w/v)	1 %	
Solution D			10 ml
Biotin	10 mg/L	0.1 mg/L	
Nicotinic acid	200 mg/L	2 mg/L	
D-Panthothenic acid	200 mg/L	2 mg/L	
Pyridoxyne hydrochloride	400 mg/L	4 mg/L	
Riboflavin	200 mg/L	2 mg/L	
Thiamine hydrochloride	200 mg/L	2 mg/L	
Adenine Solution			50 ml
Adenine $\cdot 1/2H_2SO_4$	300 mg/L	15 mg/L	
Guanine Solution *			50 ml
Guanine	592 mg/L	30 mg/L	
Trace element solution			10 ml
$CaCl_2$	109.9 mg/L	**	
$ZnSO_4$	17 mg/L	0.17 mg/L	
$CuSO_4$	8 mg/L	0.08 mg/L	
$CoCl_2 \cdot 6H_2O$	12 mg/L	0.12 mg/L	
$Na_2MoO_4 \cdot 2H_2O$	12 mg/L	0.12 mg/L	
Amino Acids Solution * **			20 ml
Glutamine Solution			10 ml
L-Glutamine	29.2 g/L	0.29 g/L	
Solution Ca			0.14 ml
$CaCl_2$	55.5 g/L	**	

* in 0.05N NaOH

** total 8.9 mg/L

*** RPMI1640 amino acids solution (50X) (Sigma, R7131)

Table 1.

Composition of CS2 medium.
CS2 was based on the HHWm medium with the modifications showing as red letters. Each stock solution was mixed (see the right column about the amount of each solution) for making CS2 medium.

Chapter 2
Figures and Tables

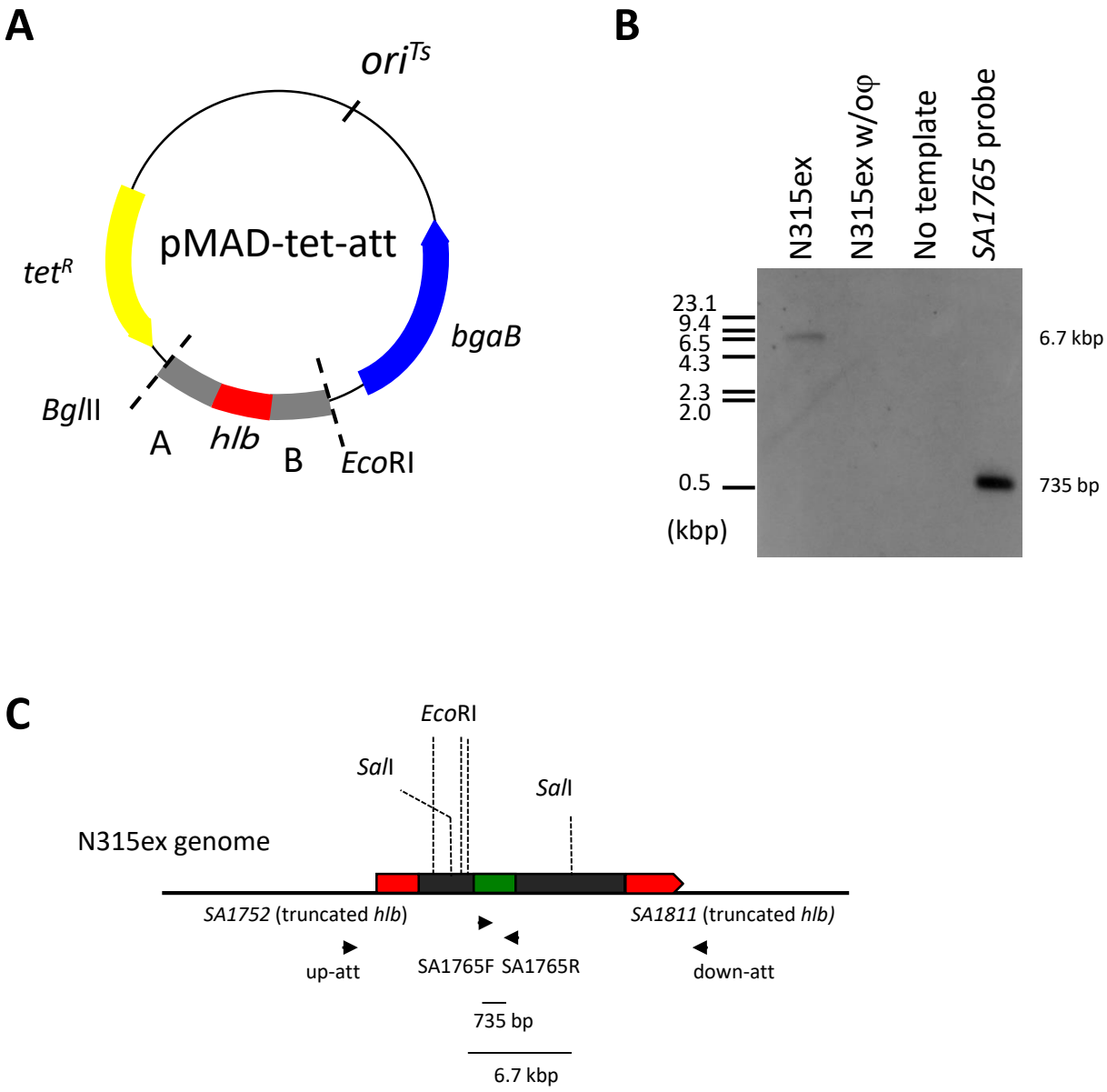

Figure 4.

Phage sequence was eliminated from N315ex genome.
(A) Map of plasmid pMAD-tet-att for construction of N315ex w/oφ. The upstream region A and down stream region B encompassing the intact *hld* sequence was inserted into *Bgl*II and *Eco*RI site of pMAD-tet. (B) Southern blot analysis with *SA1765* probe for the phage region. The genome was digested with *Sal*I and *Eco*RI. SA1765 signal was undetectable in N315ex w/oφ sample, meaning that the region was eliminated. (C) Phage locus in N315ex genome. *hlb* sequence is interpreted by phage N315. Red: Interpreted *hlb.* Green: *SA1765*. Black: phage genome.

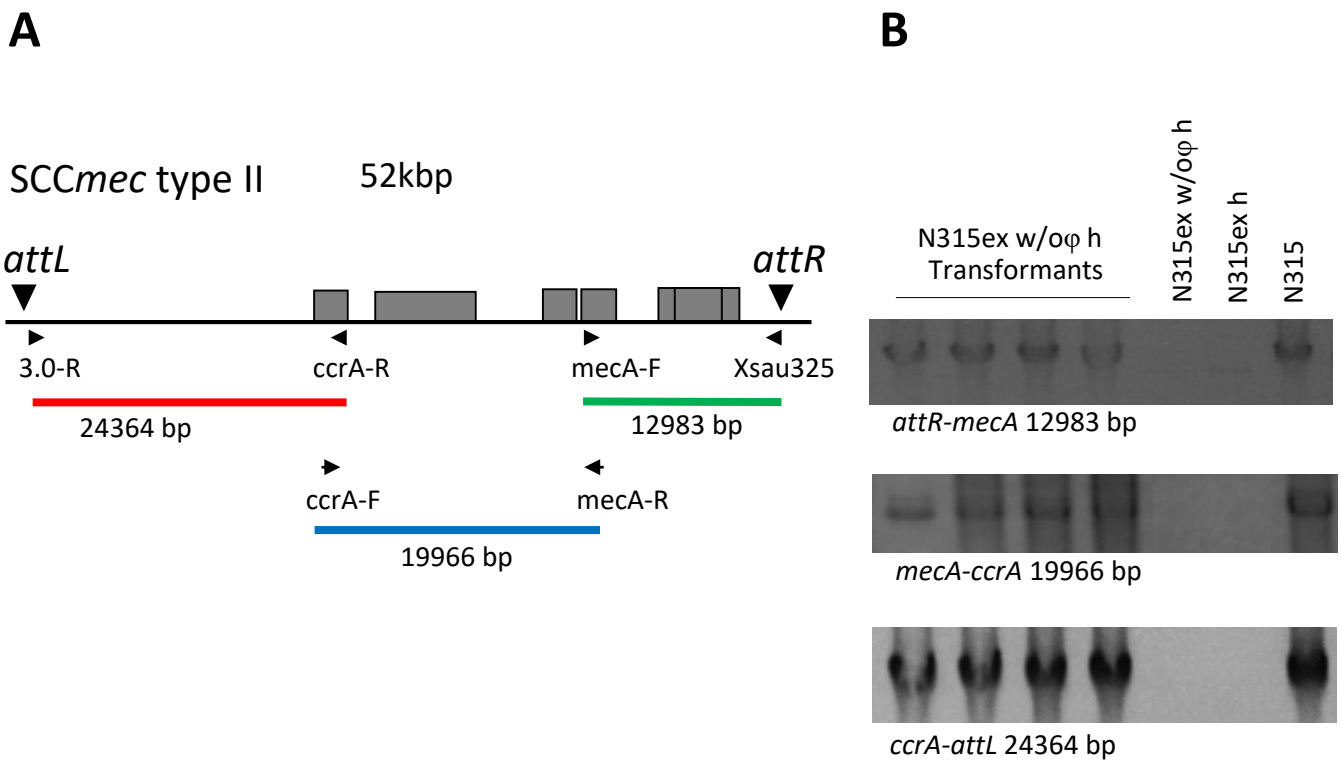

Figure 5.

SigH mediated genome transformation was independent from the lysogenic phage.

(A) Map of SCC*mec* type II. The primers are shown by allows. (B) Long-PCR analysis of transformants in phage eliminated strain. Genome transformation assay with phage-less strains was performed. Donor DNA: N315 genome that contain SCC*mec* type II. The isolated transformants by kanamycin selection were checked. N315 genome was used for positive control. Three fragments, *attR-mecA, mecA-ccrA,* and *ccrA-attL* were detected.

Strains	Description	Source
COL	MRSA, carrying tetracycline resistance plasmid pT181	McNamara PJ et al, 2008
COL w/oφ	COL strain cured of the L54a prophage	This study
N315	pre-MRSA, $Km^{R,}$ Erm^{R}	Dyke KG et al, 1966
N315h	N315 carrying pRIT-sigH	Morikawa K et al, 2003
N315ex	SCC*mec* cured derivative of N315, Km^{S}	Kuwahara-Arai K et al, 1996
N315ex-h	N315ex pRIT-sigH	Morikawa K, Takemura AJ et al, 2012
N315ex w/oφ	N315ex cured of N315 phage, Km^{S}	This study
N315ex w/oφ h	N315ex w/oφ carrying pRIT-sigH	This study
N315ex w/oφ v	N315ex w/oφ carrying pRIT5H	This study
N315ex w/oφ Δ*comE*	N315ex w/oφ Δ*comE* mutant	This study
N315ex w/oφ Δ*comE*h	N315ex w/oφ Δ*comE* pRIT-sigH	This study
N315ex w/oφ Δ*comG*	N315ex w/oφ Δ*comG* mutant	This study
N315ex w/oφ Δ*comG*h	N315ex w/oφ Δ*comG* pRIT-sigH	This study
RN4220	derivative of 8325-4, restriction minus, modification plus	Sjostrom JE et al, 1973
RKCG	RN4220 *ΔcomG* mutant, Cm^{R}	This study
RKCE	RN4220 *ΔcomE* mutant, Cm^{R}	This study
E. coli HST04 *dam*$^{-}$/*dcm*$^{-}$ pHY300	*E. coli* strain lacking the genetic factors *dam* and *dcm* that are necessary for DNA methylation, carrying pHY300PLK (Amp^{R}, Tet^{R})	Morikawa K, Takemura AJ et al, 2012

Table 2.

Strains used in chapter 2.

Name	Nucleotide sequence (5’-3’)
3.0-R	CTCAGACAGCAATTTCCCG
ccrA-F	ACGTCAAAGTACGATGAAACAAC
ccrA-R	CTGACTTGTTCTCCAATGTTATCTG
ErmA1	CACGAATATCAGTAAACAAGACAAC
ErmA2	TGCTTCAAAGCCTGTCGGAATTGGT
lena007	GGACCAATAATAATGACTAGAGAAG
lena008	CTGAAGGAAGATCTGATTGCTTAAC
mecAF	GTAGTTGTCGGGTTTGGT
mecAR	GGTATCATCTTGTACCCA
Xsaw 325	GGATCAAACGGCCTGCACA
phage F	GGAATGTACACCCCAAAAGCTAGACTGAAA
phage R	TTGCTATCATTATCGAATCCACAACCGC
1765F	CCTTGGTTGTATGTCGAAAGAGGGTTTGAA
1765R	TTTCGTGCCAGCACCAACCCAACCTTTT
up att	CGAATTCGGAACTTGATAGTTTCTTTTAGC
down att	CTAGATCTATTGGTCTGGTGAAAACCATGT

Table 3.

Oligonucleotides used in chapter 2.

Recipient Strains / Donor DNA	N315h	N315Δ*comE* h	N315Δ*comG* h	N315	N315ex w/oφ h	N315ex w/oφ v
Plasmid DNA (10 µg pT181)	$4.0 \times 10^{-9} \pm 3.0 \times 10^{-9}$ (n = 11) ND (n = 1)	ND (n = 3)	ND (n = 3)	ND (n = 3)		
Plasmid DNA (10 µg pHY300)					$2 \times 10^{-6} \pm 6.3 \times 10^{-7}$ (n = 3)	ND (n = 2)

Table 4.

SigH mediated transformation does not require phage components.
Transformation frequencies: the number of transformants / cfu of recipient strain; mean ± SD. ND: none detected (< 2×10^{-10}). Purified plasmid pT181 and pHY300 were used as donor DNA.

	Frequency
RN4220	ND (n = 3)
RN4220 φ11	$3.9 \times 10^{-9} \pm 4.0 \times 10^{-9}$ (n = 3)
RKCG φ11	$3.3 \times 10^{-9} \pm 3.0 \times 10^{-9}$ (n = 2), ND (n = 1)
RKCE φ11	$3.7 \times 10^{-9} \pm 1.1 \times 10^{-9}$ (n = 3)

Table 5.

Phage-dependent "pseudo-transformation" is distinct from the genuine natural genetic competence.

Lysogenic φ11 in strain RN4220 could mediate "pseudo-transformation" in line with previous studies. N315 genome (carrying 5 copies of *erm* genes) was used as donor DNA. In contrast to natural competence, the *comG* or *comE* operon genes were found to be dispensable; lysogenic strains of RKCG and RKCE gave the same number of Erythromycin resistant colonies. φ11: lysogenized with phage 11. ND: none detected ($<1.5 \times 10^{-10}$). For the details, refer to the classical phage-dependent pseudo-transformation method (Pattee et al. 1975; Morikawa and Takemura et al. 2012).

Chapter 3
Figures and Tables

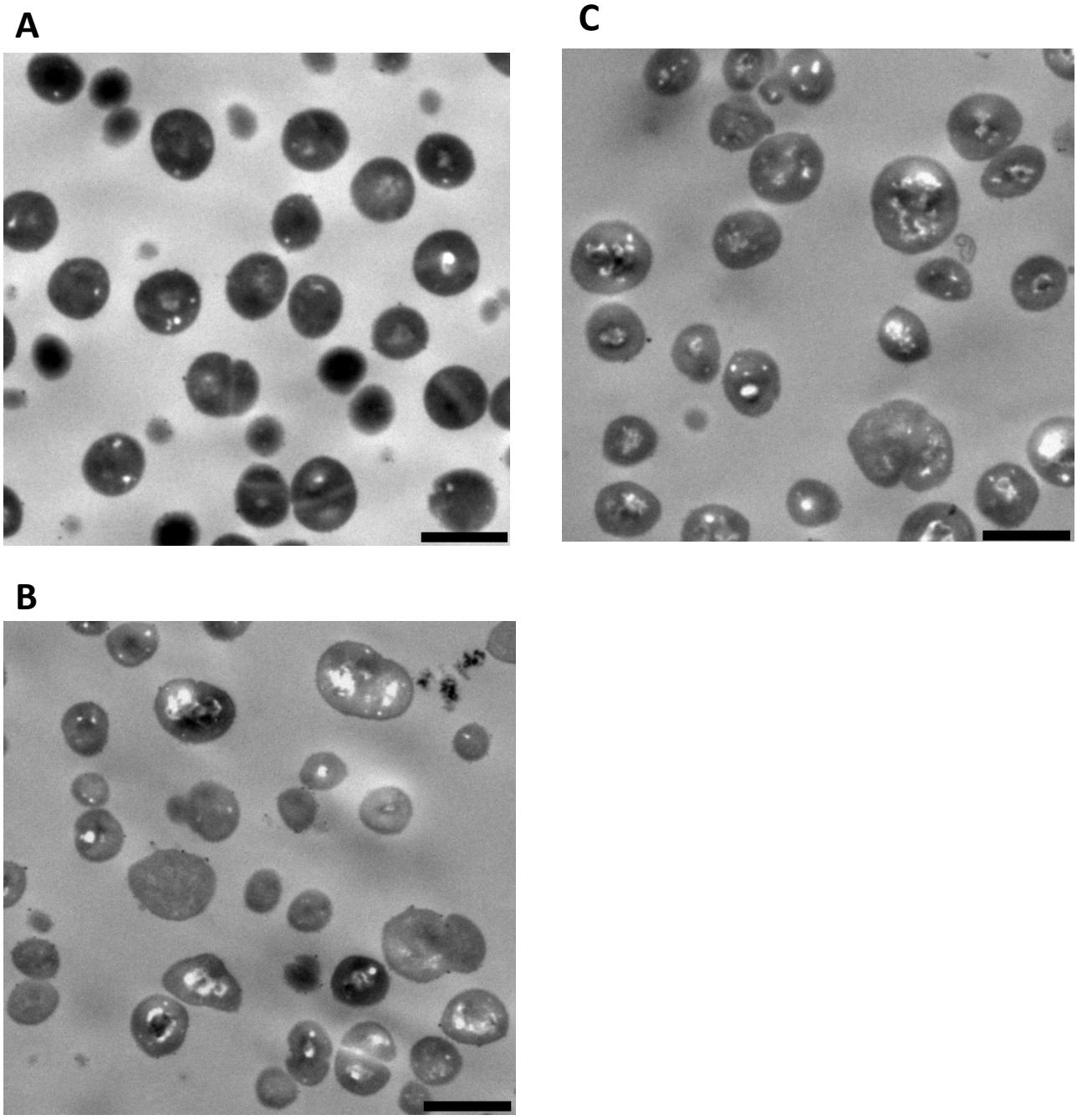

Figure 6.

Transmission electron microscopy of cells grown in CS2 medium and TSB medium.

N315 overexpressing SigH (N315 h) grown in TSB (A) and CS2 (B); (C) N315 carrying the control vector (N315 v) grown in CS2. Scale bar = 1 μm. The cell surface roughness (mean ratio ± SD, see Material and Method) in N315 h in TSB was 1.1±0.03, while those were 1.25±0.07 for N315 h in CS2 ($p < 0.01$), and 1.21 ± 0.04 for N315 v in CS2.

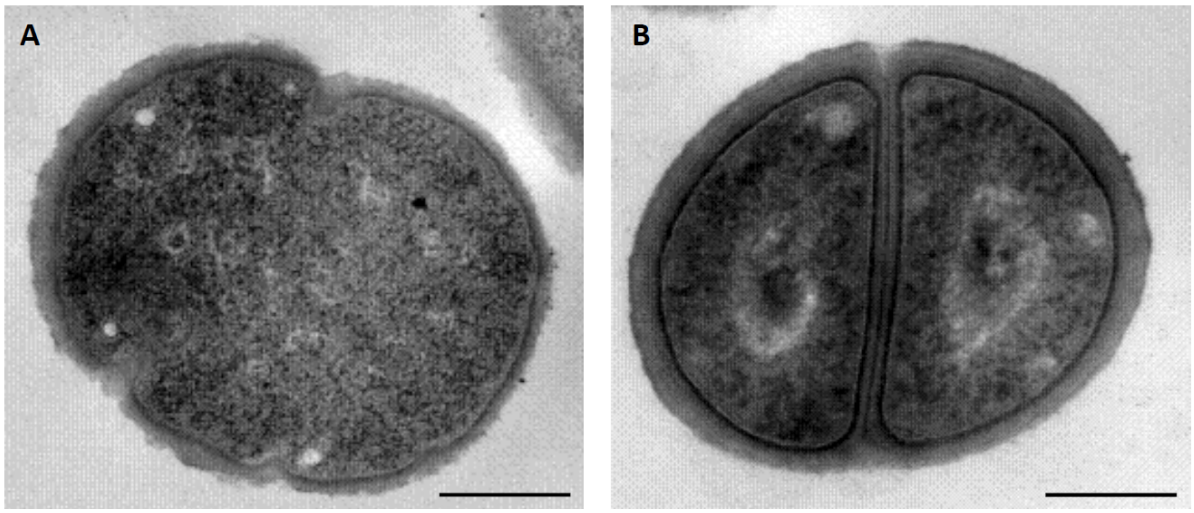

Figure 7.

Transmission electron microscopic images.
Transmission electron microscopic images of N315 (A), and COL (B). Cells were cultured for 8 hours in CS2 medium. Scale bar = 0.5 μm

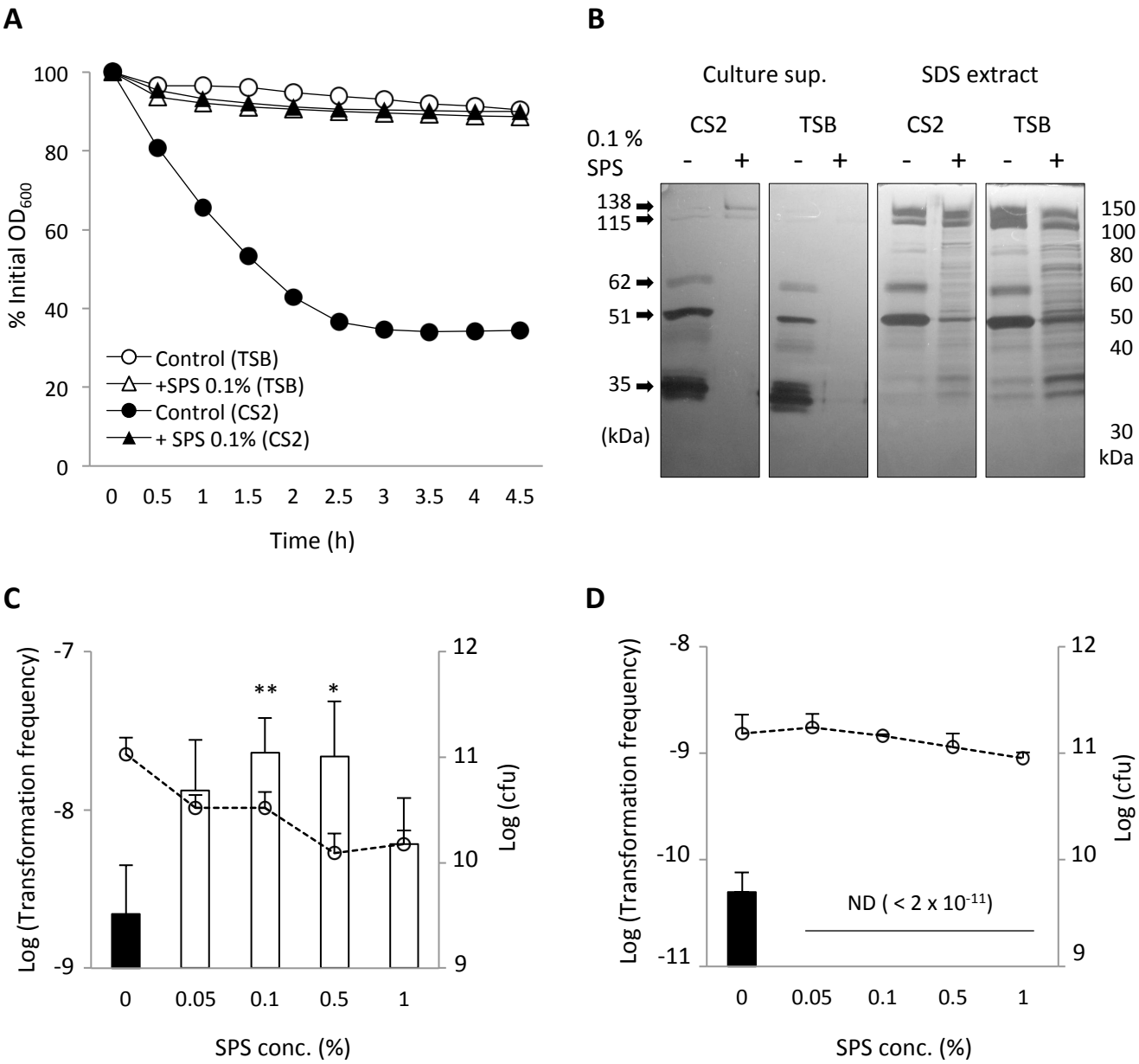

Figure 8.

Effects of autolytic activity on *S. aureus* transformation.
(A) Whole cell autolysis of cells grown in CS2 and TSB with (+) or without (−) 0.1% SPS. Data represent the averages of two independent experiments. (B) Zymogram analysis of murein hydrolases in the culture supernatant and SDS extract. Cells were grown in CS2 or TSB with or without 0.1% SPS. 138 kDa: uncleaved autolysin Atl; 115 kDa: intermediately processed Atl; 51 kDa: completely processed endo-β-*N*-acetylglucosaminidase; 62 kDa: completely processed *N*-acetylmuramoyl-l-alanine amidase; 35 kDa: LytM (Dubrac S et al. 2007). (C) Effects of SPS on transformation in CS2 medium. Mean and SD values are shown (**p<0.01; *p<0.05; n=4). (D) SPS has no positive effect on transformation in TSB medium (n=3). ND: none detected. Bars: Log_{10} (transformation frequency); dotted lines: Log_{10} (cfu).

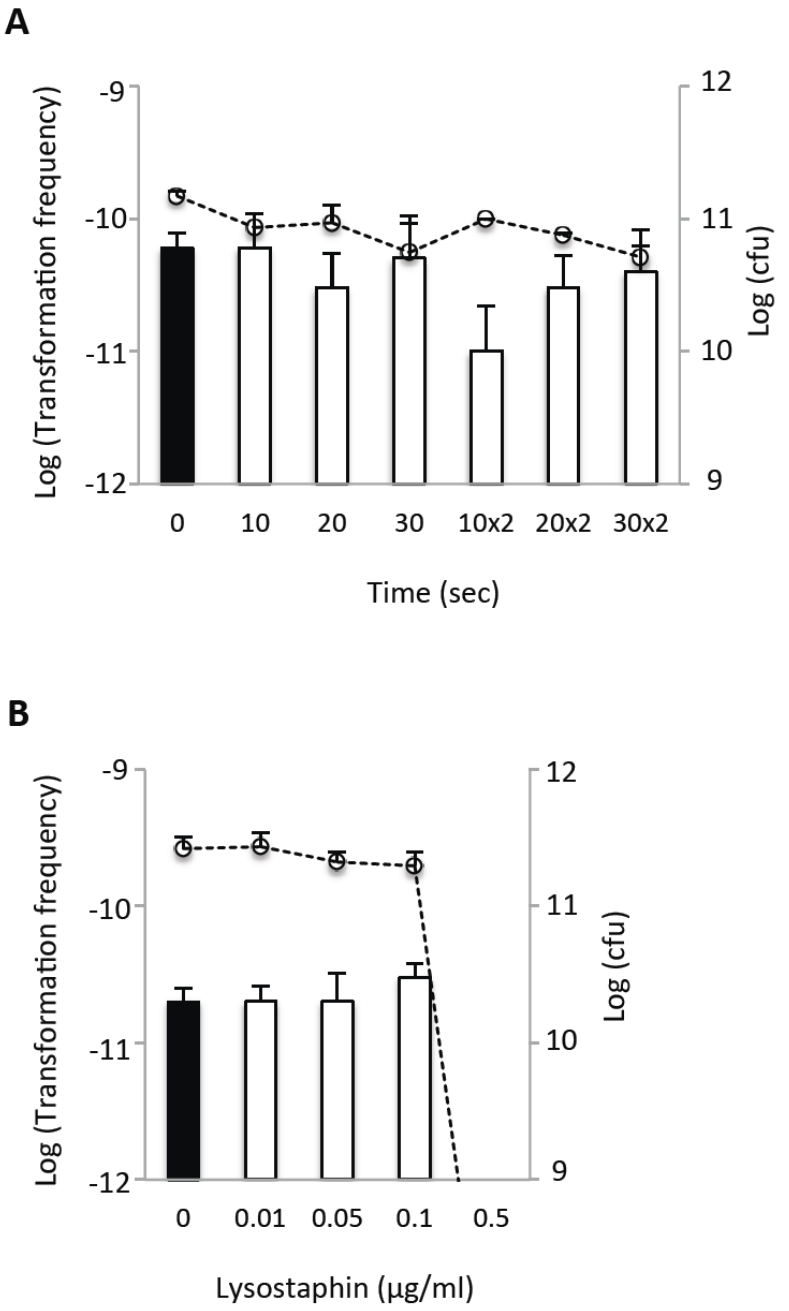

Figure 9.

Transformation frequencies in bead beating or lysostaphin treated cells.
(A) Cells were treated by Fastprep device for the indicated periods (0 sec, 10 sec, 20 sec, 30 sec and time double 10x2 sec, 20x2 sec, 30x2 sec) prior to transformation. (B) Cells were incubated with lysostaphin. The values correspond to mean and SD obtained from three independent experiments. Bars: Log_{10} (Transformation frequency); dotted lines: Log_{10} (cfu).

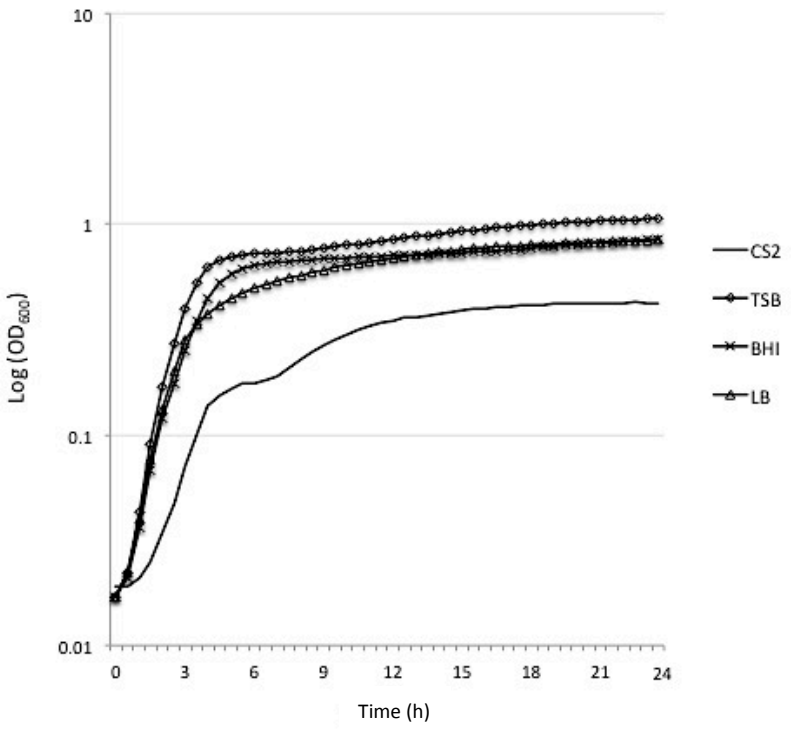

Figure 10.

Growth curves of N315ex in different media.
Cells were grown in different culture media on a 96-well plate and OD_{600} was measured with plate reader.

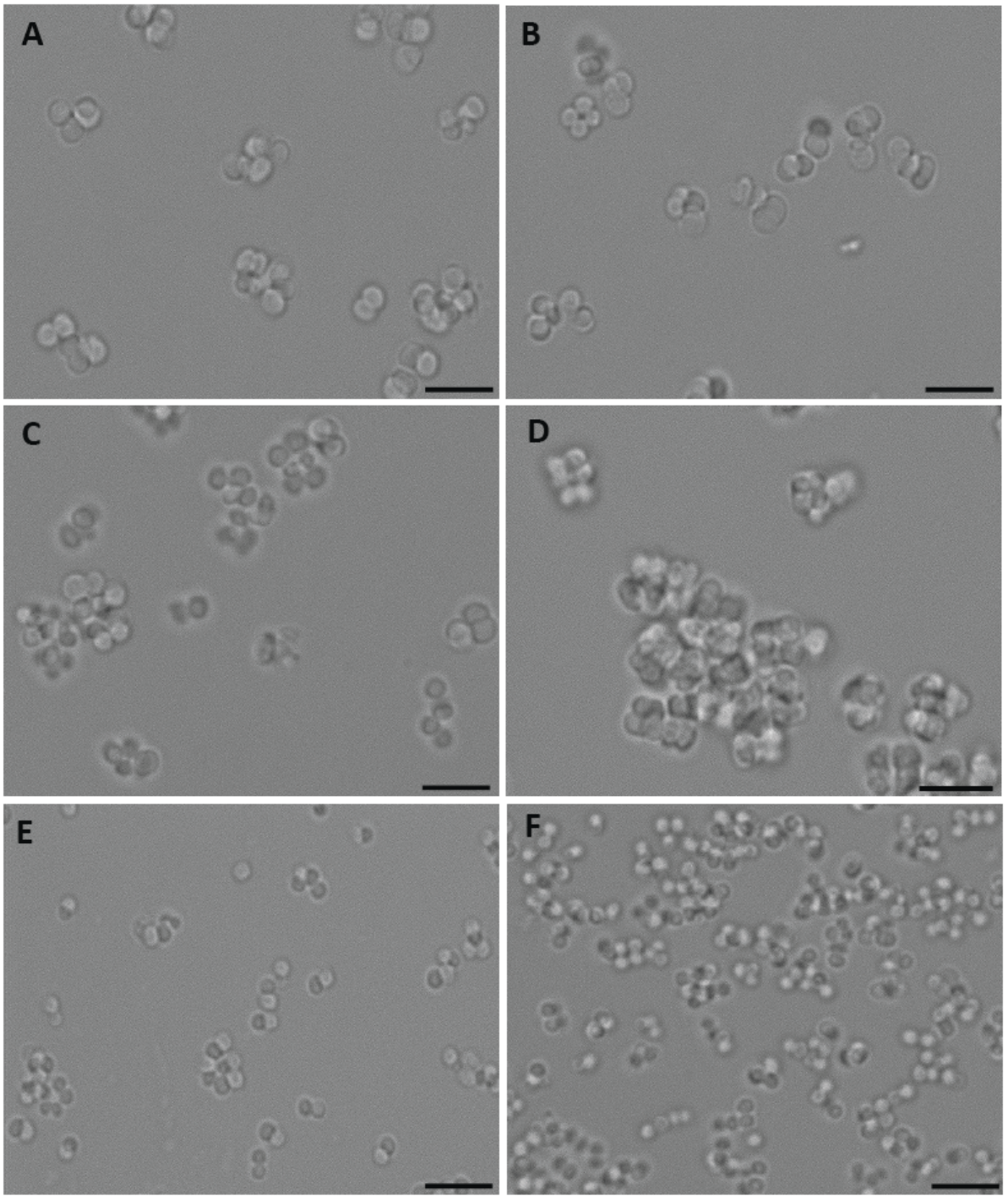

Figure 11.

Microscopy analysis.
Phase-contrast microscopic images of N315ex derivative strains cultured 8 hours in CS2 medium (A-D) or TSB (E-F) with (D, F) or without (A-C, E) 0.1% SPS. No morphology difference could be observed between N315ex-GFP (A), N315ex h-GFP (B) and N315ex Δ*sigH*-GFP (C) grown in CS2. N315ex cells grown in CS2 supplemented with 0.1% SPS (D) show cell aggregation but were not changed in cell size compared to N315ex in normal CS2 (A). No difference could be observed when N315ex was cultured in TSB with 0.1% SPS (F) compared to TSB alone (E). Scale bar = 5 μm

Strains	Description	Source
COL	MRSA, carrying pT181	Dyke KG et al, 1966
COLh	COL carrying pRIT-sigH	Morikawa K, Takemura AJ et al, 2012
N315	pre-MRSA	Kuwahara-Arai K et al, 1996
N315h	N315 carrying pRIT-sigH	Morikawa K et al, 2003
N315v	N315 carrying pRIT5H	Morikawa K et al, 2003
N315ex	SCC*mec* cured derivative of N315	Ito, T et al, 1999
N315ex-GFP	N315ex carrying pMK3-com-gfp	Morikawa K, Takemura AJ et al, 2012
N315ex h-GFP	N315ex carrying pRIT-sigH and pMK3-com-gfp	Morikawa K, Takemura AJ et al, 2012
N315ex Δ*sigH*-GFP	*sigH* mutant of N315ex carrying pMK3-com-gfp	This study
N315ex w/oφ	N315ex cured of the N315 prophage	Morikawa K, Takemura AJ et al, 2012
N315ex w/oφ h	N315ex w/oφ carrying pRIT-sigH	Morikawa K, Takemura AJ et al, 2012
N315ex w/oφ Δ*comG* h	N315ex w/oφ Δ*comG* pRIT-sigH	Morikawa K, Takemura AJ et al, 2012
N315ex w/oφ Δ*comE* h	N315ex w/oφ Δ*comE* pRIT-sigH	Morikawa K, Takemura AJ et al, 2012
E. coli HST04 *dam*$^{-}$ /*dcm*$^{-}$ pHY300	*E. coli* strain lacking the genetic factors *dam* and *dcm* that are necessary for DNA methylation, carrying pHY300PLK	Morikawa K, Takemura AJ et al, 2012
Plasmids		
pHY300PLK	Shuttle vector, ori-pAMa1, Amp^{R} (*E.coli)*, Tet^{R} (*S. aureus*)	Takara, Japan
pT181	*tetK* tetracycline resistance plasmid from COL	Dyke KG et al, 1966
pMADtetsigH	Vector for deletion of *sigH*, Amp^{R} (*E.coli)*, Erm^{R}, Tet^{R} (*S. aureus*)	This study

Table 6.

Bacterial strains and plasmids used in chapter 3.

Medium / Donor DNA	TSB	BHI	$NBCaCl_2$	CS2
Plasmid DNA pT181	ND (n = 2)	ND (n = 2)	ND (n = 2)	$4.0 \pm 3.0 \times 10^{-9}$ (n = 11)
Plasmid DNA pHY300	$5.0 \pm 4.7 \times 10^{-11}$ (n = 2) ND (n = 2)	$5.0 \pm 6.0 \times 10^{-11}$ (n = 2)	$3.0 \pm 1.4 \times 10^{-11}$ (n = 2)	$1.6 \pm 1.4 \times 10^{-9}$ (n = 8)

Table 7.

Transformation frequencies of N315ex w/oφ carrying SigH expression plasmid in different media.

Transformation frequencies were checked under the various mediums, TSB, BHI, $NBCaCl_2$, and CS2. Purified plasmid pT181 and pHY300 were used as donor DNA. Frequencies: the number of transformants / cfu of recipient strain; mean ± SD. ND: none detected (c.a. $<10^{-11}$). This indicate that environmental conditions are important for SigH derived transformation.

Chapter 4
Figures and Tables

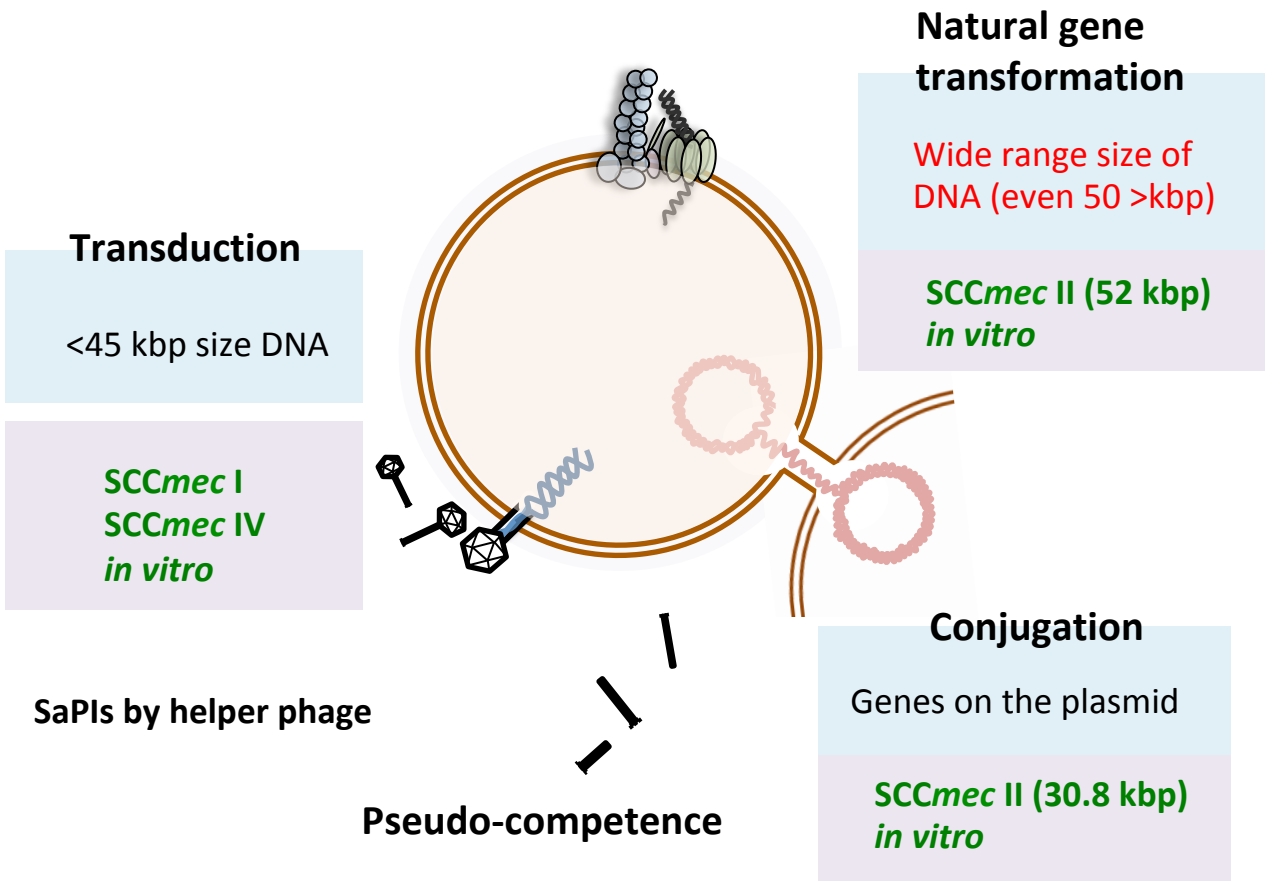

Figure 12.

Summary of *S. aureus* HGT systems

This work reveals that *S. aureus* can develop the natural transformation with the ability to uptake various sizes of DNA. Transduction by typical staphylococcal phage is though to transfer DNA under 45 kbp, since the DNA has to be packed into the phage head. Size of SaPIs that requires helper phage is around 16 kbp (see Novick et al. 2010). Conjugation allow to transfer SCC*mec* type II, however, not whole size was transferred (see Ray et al. 2016). In contrast, the natural transformation was able to transfer whole size of 52 kbp SCC*mec* type II.

The natural transformation in *S. aureus*

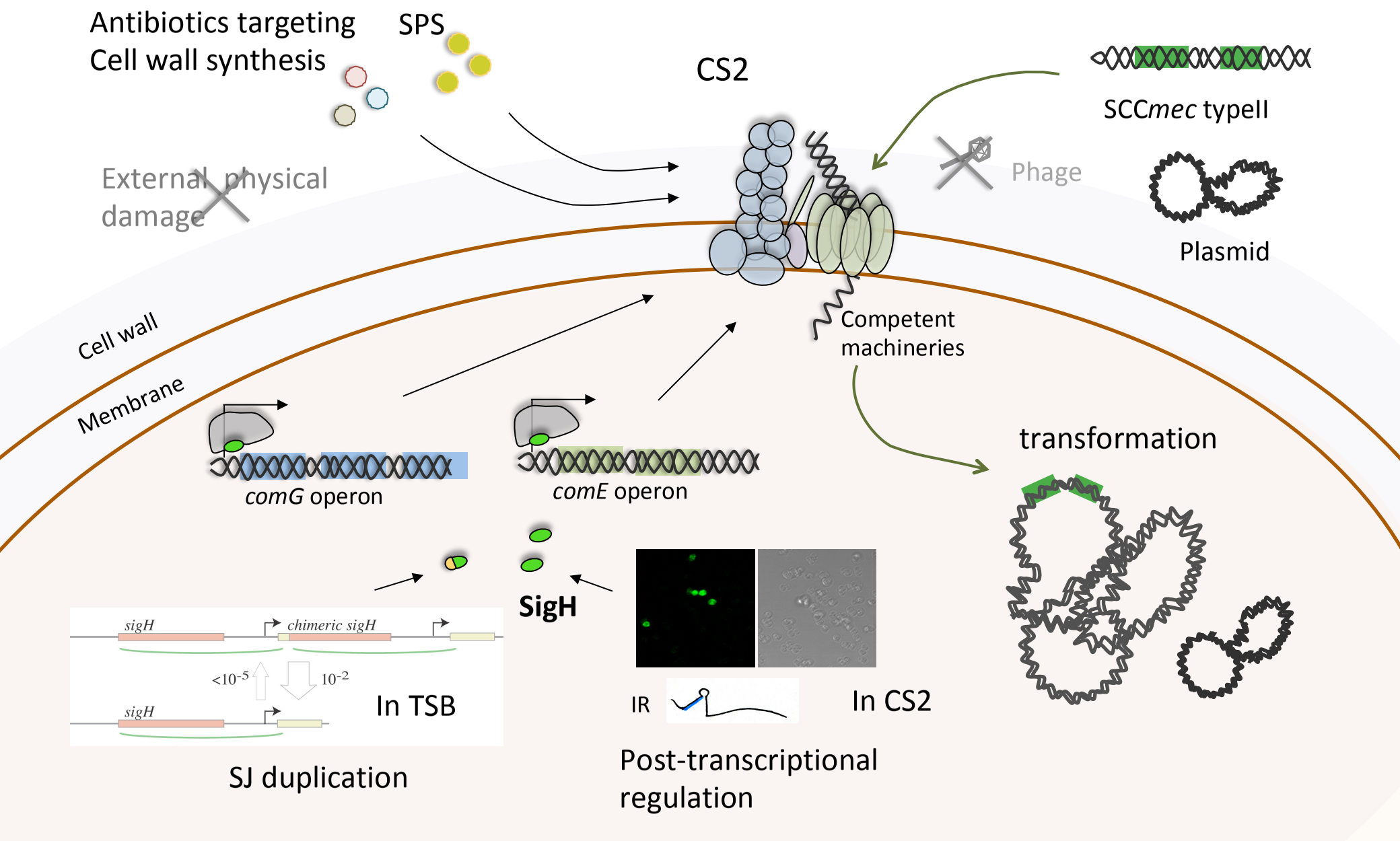

Figure 13.

Summary of the natural transformation in *S. aureus.*

S. aureus can develop the natural transformation by expressing the sigma factor, SigH, via two mechanisms; SJ duplication and post-transcriptional regulation. The transformation occur with genomic DNA and plasmid, without any phage factors (Chapter 2).

The fact that transformation is dependent on CS2 medium strongly suggests the involvement of inducing signals. SPS and antibiotics targeting cell wall synthesis affect the transformation frequency, suggesting the possible involvement of the cell wall metabolism (Chapter 3). Simple external physical damage does not facilitate the transformation.